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Is There VDAC in Cell Compartments Other than the Mitochondria?

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Higher eukaryotes, including mammals and plants, express a family of VDAC proteins each encoded by a distinct gene. Two human genes encoding VDAC isoforms (HVDAC1 and HVDAC2) have been characterized in greatest detail. These genes generate three proteins that differ primarily by the addition of distinct N terminal extensions in HVDAC2 and HVDAC2', a splice variant of HVDAC2, relative to HVDAC1. Since N terminal sequences have been demonstrated to target many proteins to appropriate subcellular compartments, this observation raises the possibility that the N terminal differences found in HVDAC isoforms may lead to targeting of each protein to different cellular locations. Consistent with this hypothesis, a large number of reports have provided evidence consistent with the notion that HVDAC1 and its homolog in related mammalian species may specifically be present in the plasma membrane or other nonmitochondrial cellular compartments. Here, we review this information and conclude that if VDAC molecules are present at nonmitochondrial locations in mammalian cells, these are unlikely to be the known products of the HVDAC1 or HVDAC2 genes.

KEY WORDS: Mitochondria; VDAC; mammals; plasma membrane; human.

INTRODUCTION

The voltage-dependent anion channel (VDAC) of the outer mitochondrial membrane, also known as mitochondrial porin, is a small abundant protein found in mitochondria from cells of all eukaryotic kingdoms (Colombini, 1989; Sorgato and Moran, 1993). It was first identified in mitochondrial fractions of *Paramecium aurelia* by Schein *et al.* (1976) as these investigators were searching for voltage-dependent calcium channel activity. Subsequently, VDAC channel activity was identified in mitochondria by subcellular fractionation (Schein *et al.*, 1976). Mitochondria from a wide variety of eukaryotes such as plants, humans, *Neurospora crassa,* and yeast have all been shown to have VDAC channels with properties that are evolutionarily conserved (for review, see Colombini *et al.*, in press). These channels share a similar (1) single channel conductance (about 4 to 4.5 nS in 1 M KCl), (2) ion selectivity (about 2:1 preference for Cl/K), and (3) voltage dependence. Further, all VDAC channels are symmetrical with respect to gating properties. When no voltage is applied, VDAC channels are in their high-conducting, open state. When voltage is increased to about 20–30 mV (both positive and negative potentials), channels switch to lower conducting, closed states. These properties characterize VDAC channels and distinguish them from all other channels observed following reconstitution into planar lipid bilayers.

VDAC is traditionally thought of as localized exclusively in mitochondria where it functions to control small-molecule (e.g., ADP, ATP) traffic between the mitochondria and cytoplasm. Recently however, a number of reports have suggested the presence of mammalian VDAC proteins in nonmitochondrial cell compartments such as the plasma membrane. The purpose of this brief review is, first, to summarize what is known about the different mammalian VDAC genes

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by focusing on human genes which encode VDAC isoforms and, second, to summarize evidence which suggests that in mammalian cells, VDAC may be present in cellular compartments other than the mitochondria.

MULTIPLE MAMMALIAN VDAC ISOFORMS

The first complete amino acid sequence of a mammalian VDAC protein was obtained by direct peptide sequencing of a protein (Porin 31HL) purified from the transformed human B-lymphocytes H2LCL cell line (Thinnes *et al.*, 1989). Using this amino acid sequence information, a full-length cDNA clone encoding this protein (referred to as HVDAC1 for the rest of this review) was identified in a human pituitary cDNA library (Blachly-Dyson *et al.*, 1993). A second human cDNA encoding a VDAC-like gene was identified by probing a human liver cDNA library at low stringency with sequences representing most of the coding region of the HVDAC1 cDNA. The resulting full-length cDNA clone encodes a protein (HVDAC2) which is 75% identical to HVDAC1 with most differences resulting from conservative changes (Blachly-Dyson *et al.*, 1993). However, in contrast to HVDAC1, the HVDAC2 sequence contains two potential translational initiation points; the first contains a good match to consensus translational initiation sequences and the second corresponds to the position of the proposed start codon in HVDAC1 and the start codons of *N. crassa* and *S. cerevisiae* genes. Use of the most 5′ start site would generate an HVDAC2 protein with an additional 11 amino acids at its N terminus compared to HVDAC1. Expression of these cDNAs in both yeast and mammalian cells has demonstrated that the native HVDAC2 protein contains this 11 amino acid N-terminal extension relative to HVDAC1 (Yu *et al.*, 1995), indicating that the most 5′ translational start site is exclusively used. Analysis of the expression of HVDAC1 and HVDAC2 by both Northern blot and polymerase chain reaction (PCR) techniques suggests that each is expressed ubiquitously. Bovine (Dermietzel *et al.*, 1994), rat (Bureau *et al.*, 1992), and mouse (Craigen *et al.*, 1994) homologs of HVDAC1 and HVDAC2 have also been identified.

A putative splice variant of HVDAC2, HVDAC2′, has also been serendipitously identified in a subtracted cDNA library (tonsillar B lymphocyte minus blood T lymphocyte) (Ha *et al.*, 1993). The initial report describing HVDAC2′ suggested that the protein encoded by this cDNA differs from HVDAC2 in both extreme N- and C-terminal domains. However, C-terminal differences are due to sequencing errors in the nucleotide sequence reported for HVDAC2′ (E. Blachly-Dyson and M. Forte, unpublished observations). Thus, HVDAC2′ is predicted to differ from HVDAC2 only in extreme N-terminal domains. Similar to HVDAC2, cDNAs encoding HVDAC2′ also contain two potential translational initiation sites, use of the most 5′ encoding a protein of 36 kDa containing a 26 amino acid extension replacing the 11 amino acid extension normally found in HVDAC2 and use of the second resulting in a protein similar in size (32 kDa) to HVDAC1. *In vitro* translation of the HVDAC2′ cDNAs (Ha *et al.*, 1993) and expression of HVDAC2′ by transient transfection of mammalian COS7 cells (Yu *et al.*, 1995) results in the production of both 32- and 36-kDa proteins, suggesting that each start codon can be used in mammalian cells. Confirmation of this notion awaits the development of antibodies which specifically distinguish HVDAC2′ from related HVDAC isoforms. Expression in yeast, however, results only in the production of the 36-kDa HVDAC2′ form, indicating that only the most 5′ translational start site is used in these cells (Yu *et al.*, 1995). Transcripts encoding HVDAC2′ cDNA also appear to be ubiquitously expressed (Ha *et al.*, 1993). Homologs of HVDAC2′ have not been identified in other mammalian organisms.

The location of genes encoding HVDAC1 and HVDAC2 within the human genome has been mapped by PCR amplification and restriction analysis of a human/rodent somatic cell mapping panel and fluorescence *in situ* hybridization. These studies demonstrate that the gene encoding HVDAC1 is located on the X-chromosome and the HVDAC2/HVDAC2′ gene is located on chromosome 5 (Blachly-Dyson *et al.*, 1994). In the process of mapping these genes, two additional HVDAC1-like genes were identified (Blachly-Dyson *et al.*, 1994). One gene mapped to chromosome 12 (HVDAC3) and was found to have 95% nucleotide sequence identity to HVDAC1 between primers used for PCR amplification. In addition, this gene has a stop codon within the coding region relative to HVDAC1, suggesting that it could represent a pseudogene. A fourth gene (HVDAC4) mapped to chromosome 1 and was also found to be 95% identical to HVDAC1 at the nucleotide level. Unfortunately, characterization of HVDAC3 and HVDAC4 has not progressed further. Multiple VDAC

genes have also been identified in mice (Craigen *et al.*, 1994).

VDAC IN CELL COMPARTMENTS OTHER THAN THE MITOCHONDRIA

The results outlined above indicated that multiple VDAC genes are present in mammals which, at least in humans, can potentially encode up to 5 isoforms of the VDAC protein. Two human genes, HVDAC1 and HVDAC2, appear to encode at least three distinct proteins which differ most significantly over their initial N-terminal regions. Since N-terminal domains often serve to target proteins to distinct intracellular compartments, these results have led to a hypothesis which suggests that the distinct N termini present in HVDAC1, HVDAC2, and HVDAC2′ lead to the targeting of these molecules to different cellular compartments. Consistent with this idea, a number of lines of evidence suggest that VDAC-like channel activity and VDAC proteins may also exist in cell compartments other than the mitochondria.

Electrophysiological Studies

Electrophysiological experiments indicate that VDAC-like channels are present in the plasma membrane (Blatz and Magleby, 1983; Jalonen *et al.*, 1989; Dermietzel *et al.*, 1994). Blatz and Magleby used single-channel, patch-clamping techniques to record a novel Cl^- channel in the plasma membrane of embryonic rat skeletal muscle cells. Experiments were performed on both inside-out and outside-out patches. This channel was selective for Cl^- over cations and has a large single-channel conductance of ~0.4 nS in 143 mM KCl. This conductance is similar to that of the mammalian mitochondrial VDAC channel at this salt concentration (0.4–0.5 nS in 0.1 M KCl) (Freitag *et al.*, 1982; De Pinto *et al.*, 1989, 1991). In addition, the channel is active at 0 mV and inactive at both negative or positive membrane potentials, again similar to the properties of mitochondrial VDAC channel. This channel was observed in less than 5–10% of membrane patches examined (Blatz and Magleby, 1983). The same frequency of occurrence of a similar channel was observed in cultured rat astrocytes although older astrocytes have higher frequency (around 30% for 9–11 week old cells) (Jalonen *et al.*, 1989). Kolb and colleagues recorded anion channels of large conductance in patch-clamp studies of channels present in the plasma membrane of transformed B lymphocytes (H2LCL cells; reviewed by Thinnes, 1992). VDAC-like channel activity has also been identified in vesicles prepared from rough endoplasmic reticulum following fusion to planar lipid bilayers (Simon and Blobel, 1991). Single channels with a conductance of 220 pS were observed in 50 mM salt solution, a conductance similar to that of mammalian VDAC at this salt concentration although the voltage sensitivity of this channel remains to be investigated.

Immunocytochemical Studies

Polyclonal antibodies have been raised against the complete human VDAC molecule purified from human B lymphocytes (HVDAC1) and against a synthetic peptide corresponding to the 19 amino acids found at the N terminus of this protein. Testing of a variety of peptides has demonstrated that antibodies directed to the N terminus only recognize peptides in which the N-terminal residue is acetylated. These antibodies were used in indirect immunofluorescence studies of both living and fixed H2LCL cells and each was reported to specifically label the plasma membrane (Thinnes *et al.*, 1989). In the case of these polyclonal antibodies, labeling of specific intracellular structures is observed but these are reported to differ from mitochondria as visualized by mitochondria-specific fluorescent stains.

In addition, eight monoclonal antibodies against HVDAC1 have also been generated. All eight monoclonal antibodies reacted equally and specifically with peptides representing the N-terminus of HVDAC1 and four were reported to specifically stain the plasma membrane of human skeletal muscle (Babel *et al.*, 1991). This set of monoclonal antibodies was also used to assess the location of this protein in a variety of cell types by indirect immunofluorescence (König *et al.*, 1991). EBV-transformed human B lymphocytes showed positive plasma membrane staining, as did several normal human B and T cell lines (König *et al.*, 1991). These results suggest that the presence of HVDAC1 on the plasma membrane is not the result of cell transformation. In addition to light level immunocytochemical studies, these monoclonal antibodies were also used at the electron microscopic level to demonstrate the presence of HVDAC1 in the plasma membrane (Cole *et al.*, 1992). In these studies, living acute lymphoblastic-leukemia cells (KM3 cell line)

were incubated with primary monoclonal antibody and FITC or gold particle conjugated secondary antibody. The labeled cells were then either examined by light microscopy or fixed, embedded, and examined by electron microscopy. At both levels, HVDAC1 appeared to be present in the plasma membrane. In these reports, no significant mitochondrial labeling by these monoclonal antibodies at either light level or electron microscopic level is reported. More recently, however, immunoelectron microscopy studies indicate that these monoclonal antibodies also label isolated rat heart mitochondria (Konstantinova *et al.*, 1995).

Studies in Astrocytes

Dermietzel and colleagues (Dermietzel *et al.*, 1994) purified a VDAC-like protein present in detergent-resistant membrane fractions prepared from bovine brain homogenates. A partial cDNA clone encoding this protein was obtained from a brain cDNA library using primers generated from peptide sequences obtained from the purified protein. The amino acid sequence of the protein encoded by this cDNA indicates that this clone encodes the bovine homolog of HVDAC1. Since the purified bovine protein cross-reacts with monoclonal antibodies recognizing the N terminus of HVDAC1, these monoclonals were used to examine the subcellular location of a protein which appears to cross-react with these monoclonals in rat astrocytes. Immunolocalization at both the light and E.M. level indicated that the cross reacting protein is specifically associated with the plasma membrane of rat astrocytes. In addition, VDAC-like channel activity was identified by patch clamp recording of the astrocyte plasma membrane. This channel has characteristics similar to mitochondria VDAC in bilayers: a single-channel conductance of 434 pS in 143 mM KCl, anion selectivity, and channel opening at low transmembrane voltage. Channels with these characteristics were observed in a subset of patches examined and only found in excised patches. Addition of the same monoclonal antibody used in immunolocalization studies to the incubation buffer blocked channel activity (Dermietzel *et al.*, 1994). Together, these results suggest the presence of the bovine and rat homologs of HVDAC1 in the plasma membrane of astrocytes, although a clear relationship between the protein purified and cloned from bovine brain, the protein in rat astrocytes that cross-reacts with monoclonals generated to HVDAC1, and the channel recorded in these cells has yet to be directly established.

Co-purification of VDAC with Proteins Normally Found in Plasma Membrane

VDAC from rat brain has been reported to copurify with the γ-aminobutyric acid type A ($GABA_A$)/benzodiazepine receptor on a benzodiazepine affinity column (Bureau *et al.*, 1992). The $GABA_A$ receptor from mammalian brain is a hetero-oligomer consisting of four or five subunits. Genes encoding subunits α, β, γ, and δ have been cloned and each exists as a family of several subtypes (Olsen and Tobin, 1990; Sigel *et al.*, 1990; Verdoorn *et al.*, 1990). Bureau and colleagues observed that in addition to these polypeptides, additional proteins appear to be present in rat brain $GABA_A$ receptor preparations prepared by benzodiazepine affinity chromatography. These accessory proteins, including the protein described below, can be removed from receptor preparations by additional chromatographic steps. Partial protein sequence for two fragments of a 36-kDa polypeptide present in these receptor preparations allowed the isolation of cDNA clones from a rat hippocampal library. This cDNA encodes a rat homolog of HVDAC2 that contains several amino acid differences from the sequence of peptides used to generate probes; the peptide sequences more closely resemble HVDAC1. The purified 36-kDa polypeptide forms a channel in lipid bilayer membranes with properties identical to that of VDAC isolated from a variety of mitochondria. An antiserum raised against purified 36-kDa polypeptide was able to adsorb purified $GABA_A$ receptor protein to some extent as assayed by [^{3}H] muscimol binding (Bureau *et al.*, 1992); 60% of the binding activity is absorbed when undiluted antisera was used and 30% absorbed with a 1:10 diluted of this antisera.

VDAC has also been reported to copurify with plasma membrane specializations such as caveolae (Lisanti *et al.*, 1994). Caveolae are 50–100-nm membrane domains representing a subcompartment of the plasma membrane. Caveolin is a 22-kDa integral membrane protein which is an important structural component of caveolae. Lisanti and colleagues developed a procedure for isolating caveolin-rich membrane domains from cultured cells. Proteins found in these enriched membranes were characterized by microsequencing and one identified as the rat homolog of HVDAC1 (Lisanti *et al.*, 1994).

Hexokinase Binding

Physiologically, VDAC is thought to function as the primary pathway for the movement of adenine nucleotides and other metabolites in and out of mitochondria, thus controlling the traffic of these essential compounds and the entry of other substrates into a variety of metabolic pathways. Results from several laboratories have shown that the VDAC protein is also the binding site of hexokinase and glycerol kinase to mitochondria (Fiek *et al.*, 1982; Lindén *et al.*, 1982; Nakashima *et al.*, 1986). For example, VDAC has the same molecular weight as the hexokinase binding protein. Both VDAC and the hexokinase binding protein have the same proteinase digestion pattern (Lindén *et al.*, 1982). VDAC is also the major mitochondrial protein which binds to [^{14}C] dicyclohexylcarbodiimide (DCCD) at relatively low dosage (2 nM of DCCD/mg of mitochondrial protein). Treatment of intact mitochondria with DCCD results in an almost complete inhibition of their ability to bind hexokinase. Fifty percent inhibition of binding occurs at less than 2 nM of DCCD/mg of mitochondrial protein (Nakashima *et al.*, 1986). Evidence directly demonstrating that VDAC and the hexokinase binding protein are the same comes from the work of Fiek and colleagues in which the hexokinase binding protein and mitochondrial VDAC were isolated from rat liver mitochondria by different procedures (Fiek *et al.*, 1982). Reconstitution of the purified hexokinase-binding protein into lipid vesicles made these vesicles permeable to ADP and generated the formation of asymmetric pores in lipid bilayers identical to those obtained from VDAC.

A number of additional studies suggest that binding of these enzymes may occur specifically to VDAC localized at contact sites between the inner and outer mitochondrial membrane (Kottke *et al.*, 1988; Brdiczka and Wallimann, 1994). Thus, binding of enzymes like hexokinase to contact sites of mitochondria has been proposed to link cytoplasmic metabolism and ADP production with the regulation of mitochondrial respiration in the mitochondrial matrix (Polaskis and Wilson, 1985; Kottke *et al.*, 1988; Brdiczka, 1990). This binding may facilitate the access of enzymes to ATP generated within the mitochondria and regulate metabolism (for review, see Adams *et al.*, 1991), although this view has recently been questioned (Kabir and Nelson, 1991).

Of the four isoforms of hexokinase expressed in mammalian cells, the hexokinase 1 (HK1) isoform appears to be specifically associated with mitochondria (Kottke *et al.*, 1988). The ability of HVDAC1 and HVDAC2 to bind HK1 has been assessed following expression of each cDNA in yeast cells which do not contain the endogenous yeast VDAC gene (Blachly-Dyson *et al.*, 1993). When compared with the binding of hexokinase to yeast mitochondria lacking VDAC, the results show that mitochondria expressing HVDAC1 are capable of specifically binding hexokinase, whereas mitochondria expressing HVDAC2 only bind hexokinase at background levels. This difference in hexokinase binding is the only known functional difference between HVDAC1 and HVDAC2.

The binding of hexokinase and glycerol kinase to mitochondria is dynamic, varying in tissues, developmental stage, and metabolic state (for review, see Adams *et al.*, 1991). For example, in brain about 80% of hexokinase is mitochondrial bound, whereas in liver the amount is considerably lower (10–20%) and depends on metabolic state. In contrast to the adult, 90% of human fetal liver glycerol kinase is bound to mitochondria (for review, see Brdiczka, 1990). Transformed liver cells tend to show an increased dependence on glycolytic metabolism for energy production and have a large increase in hexokinase activity compared to normal cells (Burk *et al.*, 1967; Weinhouse, 1972; Singh *et al.*, 1974; Bustamante and Pedersen, 1977; Bustamante *et al.*, 1981; Nakashima *et al.*, 1984). Further, in normal liver cells, particulate hexokinase distributes with nonmitochondrial membrane markers while particulate hexokinase distributes with outer mitochondrial membrane markers in transformed cells. Thus, in normal glucose-utilizing tissues such as brain, hexokinase has a propensity to be localized to nonmitochondrial particulate compartments, whereas in tumor cells particulate hexokinase is associated with mitochondria by binding to VDAC (Nakashima *et al.*, 1986; Arora and Pedersen, 1988). The first 15 amino acids at the N-terminus of rat HK1 are necessary and sufficient to confer mitochondrial binding (Gelb *et al.*, 1992). Since the N-terminal 15 amino acids of HK1 isolated from c37 mouse hepatoma cell line is identical to that of HK1 associated with nonmitochondrial binding sites in normal liver cells (Schwab and Wilson, 1989; Arora *et al.*, 1990), the redistribution of HK1 from nonmitochondrial compartments in normal cells to mitochondria in transformed cells is likely due to association with different populations of receptors. Thus, if the nonmitochondria binding sites for particulate HK1 in nontransformed cells are VDAC or VDAC-like molecules as they are in mitochondria, it is possible that multiple forms of

VDAC may be required in mammals to mediate the differential localization of hexokinase, and perhaps other molecules, to different sites within the cell depending on physiological state.

DIRECT LOCALIZATION OF HUMAN VDAC ISOFORMS IN CULTURED MAMMALIAN CELLS

Human isoforms of VDAC share extensive amino acid homology (roughly 75%) and do not differ sufficiently in any one domain to allow the generation of antibodies that will distinguish each isoform. To directly determine the subcellular location of HVDAC1 and define the cellular compartments containing other members of the VDAC family of proteins, cDNAs encoding HVDAC1, HVDAC2, and HVDAC2′ were modified to incorporate sequences directing the insertion of epitopes at the extreme C-terminus of each protein (Yu *et al.*, 1995). In this recent study, two such epitopes were employed; the synthetic FLAG epitope and an epitope derived from the human influenza hemagglutinin protein (HA). The inclusion of either epitope appeared not to affect the function of these molecules since each is efficiently targeted to the yeast mitochondria and is capable of complementing growth defects associated with the elimination of the endogenous yeast VDAC gene, as are the unmodified forms of these human proteins. Since specific antibodies only for HVDAC1 have been described (see above), this system provided a convenient way to individually tag each HVDAC isoform and follow the expression of these molecules in a variety of cell types.

The targeting of individual tagged isoforms within mammalian cells was assessed following transient transfection into two different cell types. In COS7 cells, three different approaches all lead to the conclusion that each HVDAC isoform is located largely, if not exclusively, in mitochondria. First, cells individually transfected with each HVDAC isoform were lysed and crude subcellular fractions prepared by differential centrifugation. In each case, Western blots of ensuing fractions demonstrated that tagged HVDAC molecules were exclusively located in fractions containing mitochondria as identified by re-probing blots with an antibody to subunit IV of mitochondrial protein cytochrome C oxidase. Second, transfected cells were examined by indirect immunocytochemical techniques. In each case, tagged HVDAC molecules are exclusively confined to subcellular structures identified as mitochondria by the inclusion of a second antibody to the cytochrome C oxidase holoenzyme during staining. In these cases, there is essentially no plasma membrane staining of either transfected or untransfected cells. Third, immunoelectron microscopic examination of transfected cells was used to unambiguously localize HVDAC1 and HVDAC2 to mitochondria. Again, in each case epitope-tagged HVDAC molecules are located exclusively in mitochondria and no staining above background is present in plasma membrane regions or any other cellular compartment. Further immunofluorescence and immuno-E.M. studies of epitope-tagged HVDAC1 and HVDAC2 COS7 cells also indicated that both HVDAC1 and HVDAC2 can be present in the same mitochondrion. Thus, these studies in COS7 cells make it unlikely that any significant fraction of the HVDAC1, HVDAC2, or HVDAC2′ molecules expressed in these cells is present in any cellular compartment other than mitochondria.

To address possible cell-specific targeting of HVDAC isoforms to other cellular compartments, the cellular distribution of HVDAC isoforms was examined in rat astrocytes transfected with constructs encoding epitope-tagged HVDAC1, HVDAC2, and HVDAC2′ molecules. As described above, these cells have recently been reported to contain rat homologs of HVDAC1 in their plasma membranes (Dermietzel *et al.*, 1994). However, immunocytochemical analysis of transfected astrocytes has demonstrated that each epitope-tagged HVDAC molecule is located exclusively in mitochondria as again identified by inclusion of cytochrome C oxidase antibodies. In the case of astrocytes, some plasma membrane staining with anti-FLAG antibodies was evident in transfected cells. However, this staining was clearly not specific, since untransfected cells show identical levels of plasma membrane staining. In addition, differences between results obtained by Yu and colleagues and Dermietzel and colleagues could not be attributed to differences in fixation technique since identical mitochondrial staining patterns were observed regardless of cell fixation with paraformaldehyde (Yu *et al.*, 1995) or ethanol (Dermietzel *et al.*, 1994).

CONCLUSIONS

Higher eukaryotes, including mammals and plants, express a family of VDAC proteins each

encoded by a distinct gene (Bureau *et al.*, 1992; Blachly-Dyson *et al.*, 1993; Ha *et al.*, 1993; Blachly-Dyson *et al.*, 1994; Craigen *et al.*, 1994; Hains *et al.*, 1994). Two human genes encoding VDAC isoforms (HVDAC1 and HVDAC2) have been characterized in greatest detail (Blachly-Dyson *et al.*, 1993, 1994). These genes generate three proteins that differ primarily by the addition of distinct N-terminal extensions in HVDAC2 and HVDAC2′ relative to HVDAC1 (Blachly-Dyson *et al.*, 1993; Ha *et al.*, 1993). Since amino terminal sequences have been demonstrated to target many proteins to appropriate subcellular compartments, this observation raises the possibility that the N terminal differences found in HVDAC isoforms may lead to targeting of each protein to a different cellular locations. Consistent with this hypothesis, a large number of reports summarized here have provided evidence consistent with the notion that HVDAC1 and its homolog in related mammalian species may specifically be present in the plasma membrane of a number of different mammalian cell types. As summarized above, three lines of experimentation have been used to demonstrate the presence of these molecules in this cellular compartment. First, a large number of studies reporting the presence HVDAC1 in the plasma membrane of a variety of cell types depend primarily on the use of a set of monoclonal antibodies that recognize the N terminus of HVDAC1 (e.g., Babel *et al.*, 1991; König *et al.*, 1991; Cole *et al.*, 1992; Dermietzel *et al.*, 1994). In these cases, it is easy to imagine that nonspecific reactivity with plasma membranes may have been mistaken for the presence of HVDAC molecules in this cellular compartment. As shown in more recent studies (Yu *et al.*, 1995), antibodies for epitopes not found in nature (FLAG) can label plasma membranes in certain cell types, although this labeling is clearly nonspecific. Consistent with this interpretation, monoclonal antibodies to the N terminus of HVDAC1 are only able to recognize "plasma membrane" forms of VDAC if they are added to living cells prior to fixation (Cole *et al.*, 1992). In addition, polyclonal antisera to mammalian VDAC proteins have been generated by a number of other groups which fail to identify plasma membrane forms of VDAC (Lindén *et al.*, 1984; Müller *et al.*, 1994). While it is impossible to exclude the possibility that some very small fraction of the VDAC is present in the plasma membrane below the limits of detection of the three techniques used by Yu and colleagues (Yu *et al.*, 1995), these more recent results indicate that it is unlikely that any significant fraction of HVDAC1, HVDAC2, or HVDAC2′ can be in the plasma membrane and that the vast majority of each of these proteins is located in mitochondria, regardless of isoform. Second, HVDAC1 has been observed to "copurify" with proteins normally found in the plasma membrane, like the $GABA_A$ receptor, or with plasma membrane specializations such as caveolae (Kayser *et al.*, 1989; Bureau *et al.*, 1992; Dermietzel *et al.*, 1994; Lisanti *et al.*, 1994). However, in these cases, the starting material for purification is essentially a total membrane fraction including mitochondria. Since VDAC is the major protein of outer mitochondrial membranes, it is easy to imagine that outer membranes, and therefore VDAC, can contaminate these preparations. VDAC may then nonspecifically associate with other proteins during subsequent solubilization and purification. In this light, it is interesting to note that VDAC can easily be separated from "purified" $GABA_A$ receptors by subsequent chromatographic steps (Bureau *et al.*, 1992). Finally, and most compelling, a number of studies report the presence of plasma membrane channels with physiological characteristics similar to purified VDAC when incorporated into planar lipid bilayers (e.g., Blatz and Magleby, 1983; Jalonen *et al.*, 1989; Dermietzel *et al.*, 1994). In the majority of these studies, VDAC-like channels are only observed in a subset of patches following incubation of excised plasma membrane patches in rather unusual ionic conditions for extended periods of time (many minutes). If plasma membranes contain VDAC channels, these channels are unlikely to normally function in this membrane as has been observed for purified mitochondrial VDAC reconstituted into planar lipid bilayers since this would result in plasma membranes that are much more permeable than they are observed to be. Thus, excision of patches and unusual conditions may be required to record from VDAC channels which normally function within conduction pathways formed by a complex of proteins with physiological characteristics very different from mitochondrial VDAC in bilayers (Thinnes, 1992). However, in spite of these reservations, the physiological evidence for the presence of plasma membrane channels which can, under certain circumstances, have basic properties similar to mitochondrial VDAC in artificial bilayers is extensive. In addition, binding sites appear to exist in nonmitochondrial compartments for metabolic enzymes such as hexokinase and glycerol kinase which associate with mitochondria by binding to VDAC. Given the results summarized above, these VDAC-like channels in the plasma membrane and nonmitochondrial binding sites for meta-

bolic enzymes are unlikely to be due to the presence of HVDAC1, HVDAC2, or HVDAC2′ in these cell compartments. Rather, these channels and binding sites are most likely composed of proteins that remain to be identified and characterized and that may or may not be related to VDAC.

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