CLONING AND SUBCELLULAR LOCALIZATION OF HUMAN VDAC ISOFORMS

by

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ABSTRACT

The voltage-dependent anion channel of the outer mitochondrial membrane, VDAC (also known as mitochondrial Porin), is a small abundant protein which forms a voltage-gated pore when incorporated into planar lipid bilayers. This protein forms the primary pathway for movement of major metabolites through the outer membrane. VDAC genes have been cloned from a variety of eukaryotic organisms including the fungi, *Saccharomyces cerevisiae* and *Neurospora crassa,* and humans. The channel properties of VDAC are evolutionarily conserved despite a lack of primary sequence conservation. In humans, several lines of evidences indicated that multiple VDAC genes encode at least 5 VDAC isoforms. The first VDAC gene characterized, HVDAC1, was identified on the basis of amino acid sequence information obtained from protein purified from human B lymphocytes. Using probes derived from HVDAC1 cDNAs at low stringency, we cloned an additional human VDAC gene, HVDAC2, from a human liver cDNA library. HVDAC2 cDNAs encode a protein that is 75% identical to that of HVDAC1 and differs from HVDAC1 by the presence of an additional 11 amino acids at its N-terminus. Recently, another human VDAC cDNA, HVDAC2', has also been described. This transcript appears to be a splice varient of HVDAC2 and encodes a protein that differs from HVDAC2 by the use of an alternate exon encoding a distinct N terminus. HVDAC2' differs most significantly from HVDAC1 by the additional of 26 amino

acids at its N-terminus. Both HVDAC1 and HVDAC2 proteins compliment the temperature sensitive growth deficiency of *vdac*$^-$ yeast strain and form functional voltage-dependent anion channels identical to those described in other organisms when incorporated into planar lipid bilayers. HVDAC1 and HVDAC2 gene transcripts have been detected in essentially all tissues and cell lines.

The differences among the N-termini of human VDAC isoforms suggest the hypothesis that the distinct amino termini lead to the targeting of individual VDAC isoforms to different cellular compartments. Consistent with this hypothesis, recent reports suggest that HVDAC1 is found in the plasma membrane of mammalian cells. To define the subcellular location of HVDAC isoforms, HVDAC genes were modified so that the encoded proteins contain C terminal epitopes recognized by either of two monoclonal antibodies. Introduction of these epitope tags had no effect on the function of modified VDAC proteins as assessed by their ability to complement growth defects when expressed in *vdac-* yeast strains. Epitope-tagged proteins were then individually expressed in COS7 cells or rat astrocytes and the intracellular location of each isoform subsequently identified by subcellular fractionation, light level immunofluorescence, and immunoelectron microscopy. Our results demonstrate that each HVDAC protein is exclusively located in fractions or subcellular regions containing mitochondrial marker proteins. In addition,

Introduction

Mitochondria, the energy generating organelles in cells, have their own genome yet the mitochondrial genome encodes only a small portion of the proteins found in mitochondria [for example the major subunits of cytochrome oxidase and ATP synthetase (Anderson, Bankier et al. 1981; Bibb, Van Etten et al. 1981)]. Most mitochondrial proteins then, are encoded by nuclear genomic DNA and are imported from cytosol. The mechanism by which nuclearly encoded components of the mitochondria are transported from sites of translation and incorporated into forming mitochondria depends on the submitochondrial compartment to which an individual protein is targeted. Imported components of the mitochondrial matrix and intermembrane space are synthesized initially as precursor forms. Most precursors have amino-terminal sequences which target them to the mitochondria and are subsequently cleaved by specific proteinases. The targeting sequences found in the amino terminus contains hydroxylated and basic amino acids and lack acidic amino acids [for a review, see (Schwarz and Neupert 1994)]. Following synthesis and targeting to mitochondria, proteins are translocated to appropriate submitochondrial compartments by a number of mechanisms as summarized by Hannavy et al. in 1993 (Hannavy, Rospert et al. 1993). For matrix components, newly synthesized proteins in the cytosol are bound to cytosolic heat shock proteins and incorporated into mitochondria in an ATP-dependent manner following binding to the mitochondrial outer membrane receptor. Matrix proteins are then translocated across both inner and outer membrane at the contact sites between inner and outer mitochondrial membranes. Translocation across the inner membrane requires a membrane potential, ATP in the matrix and a

mitochondrial heat shock protein, mhsp70. Intermembrane space proteins are translocated into mitochondria by mechanisms similar to those used by matrix proteins. Two models have been proposed for the intramitochondrial sorting of these proteins. In one model, intermembrane space proteins are first imported into the matrix and then retranslocated across the inner membrane (Hartl, Ostermann et al. 1987). An alternative model proposes that these proteins become arrested in the inner membrane by a sorting sequence and then are directly released to the intermembrane space (Glick, Brandt et al. 1992). However, additional translocation pathways appear to be present for intermembrane space proteins. Apocytochrome C can penetrate the outer membrane without binding to receptor proteins and is trapped in the intermembrane space after the covalent attachment of heme (Nicholson, Hergersberg et al. 1988). Another intermembrane space protein, cytochrome c heme lyase, needs receptor to translocate across the outer membrane but this process is dependent neither on a membrane potential nor on extramitochondrial ATP (Lill, Stuart et al. 1992).

Outer mitochondrial membrane proteins are generally targeted directly to this membrane and are not subject to proteolytic processing during integration (Freitag, Neupert et al. 1982; Hase, Muller et al. 1984; Mihara and Sato 1985; Kleene, Pfanner et al. 1987). The targeting signal of one of the yeast outer mitochondrial membrane proteins, the 70 kD protein, has been defined at its N-terminus (Hase, Muller et al. 1984; Hase, Nakai et al. 1986). However, many of the details concerning the targeting of outer membrane components to this mitochondrial subcompartment and mechanisms associated with subsequent insertion and assembly into this membrane remain to be determined.

Basic properties of VDAC protein

Physiological properties of VDAC channel

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* (Schein, Colombini 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 studies (Schein, Colombini et al. 1976). Mitochondria from a wide variety of eukaryotes such as plants, humans, *Neurospora crassa* and yeast have all been shown to have mitochondrial VDAC-like channels (Colombini 1979) with properties that are evolutionarily conserved. As reviewed by Benz in 1985, from yeast to mammals, VDAC channels share 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 (Benz 1985; Colombini 1989). 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 the closed state. These properties characterize VDAC and distinguish them from all other channels observed following reconstitution into planar lipid bilayers.

Pore size of VDAC

The molecular weight of the VDAC protein varies from 30 kDa to 35 kDa. The highly conductive channels formed after incorporation into bilayer membranes (4 to 4.5 nS in 1M KCl) reflects a large aqueous pore. The size of

the pore was estimated by determining the size of the largest nonelectrolyte that permeates through the channel (Colombini 1980a; Colombini 1980b). In the open state, VDAC is permeable to inulin (M_{av} = 5000, R=1.4nm) and polyethylene glycol 3400 (M_{av}=3400, R=1.9nm) but not permeable to PEG 6800. These results suggest an open pore diameter of about 4 nm. In the closed state, channels are not permeable to inulin or PEG 1500 (R=1.2 nm) but are slightly permeable to gamma cyclodextrin (R=0.84). Thus, the estimated diameter of closed state is about 1.8 nm (Colombini, Yeung et al. 1987). Closed states have a reduced permeability towards ions and are cation selective rather than anion selective (Ludwig, Benz et al. 1989). Consistent with this observation, closed channels exclude adenine nucleotide permeation through the outer membrane of intact mitochondria (Colombini, Yeung et al. 1987). Electron microscopic images of negatively stained VDAC crystals embedded in vitreous ice and aurothioglucose indicated that the diameter of the open VDAC channel is in the range of 2.5-3.0 nm, consistent with the value estimated from the non-electrolyte permeation (Mannella and Tedeschi 1992).

Molecular structure of VDAC channels

Composition

VDAC genes have been cloned from mammalian, plant and fungal sources. In most cases, VDAC is composed of 283 amino acids (Mihara and Sato 1985; Kleene, Pfanner et al. 1987; Blachly-Dyson, Zambronicz et al. 1993). There is however, some variation in this basic structure since two human VDAC isoforms have additional 11 or 26 amino acids extensions at their N-termini as deduced from the nucleotide sequences (Bureau, Khrestchatisky et al. 1992; Blachly-Dyson, Zambronicz et al. 1993).

Several lines of evidences suggested that each VDAC channel is formed by one polypeptide. Examination of frozen-hydrated specimens (Mannella 1986; Mannella 1987) and scanning electron microscopy studies (Thomas, Kocsis et al. 1991) of two dimensional crystals made of *Neurospora crassa* VDAC indicate that the mass per channel-forming unit is less than needed for a dimer. Studies (Peng, Blachly-Dyson et al. 1992) in yeast also support this hypothesis. Yeast VDAC mutants with altered selectivity were generated by site-directed mutagenesis. Plasmid expressing these mutant yeast VDACs were introduced into yeast cells containing the wild type VDAC gene. If functional VDAC channels were formed by more than one polypeptide, channels whose selectivity was between that of wild-type and purely mutant channels should be observed. When channels formed by VDAC purified from such yeast cells are analyzed, only wild-type or mutant channels were present; no hybrid channels were detected. A sufficient number of channels was examined so that the probability of missing a hybrid was less than 1 in 10^7. These experiments agree with the conclusion that each VDAC channel is composed of one polypeptide.

Secondary structure

The highly conserved channel properties of VDAC require a fundamental conservation of protein structure. Yet, there is only 37% identity in the primary sequences of VDAC from *Saccharomyces cerevisiae* and *Neurospora crassa* and 24% identity between the yeast and human VDAC amino acid sequences. Despite the lack of conservation of primary amino acid sequence, the pattern of VDAC's secondary structure, as analyzed by computer algorithm, is highly conserved (Colombini, Peng et al. 1992). One analysis done by Blachly-Dyson *et al.* (1989) looked for 10 amino acid stretches of alternating polar and nonpolar residues capable of producing transmembrane

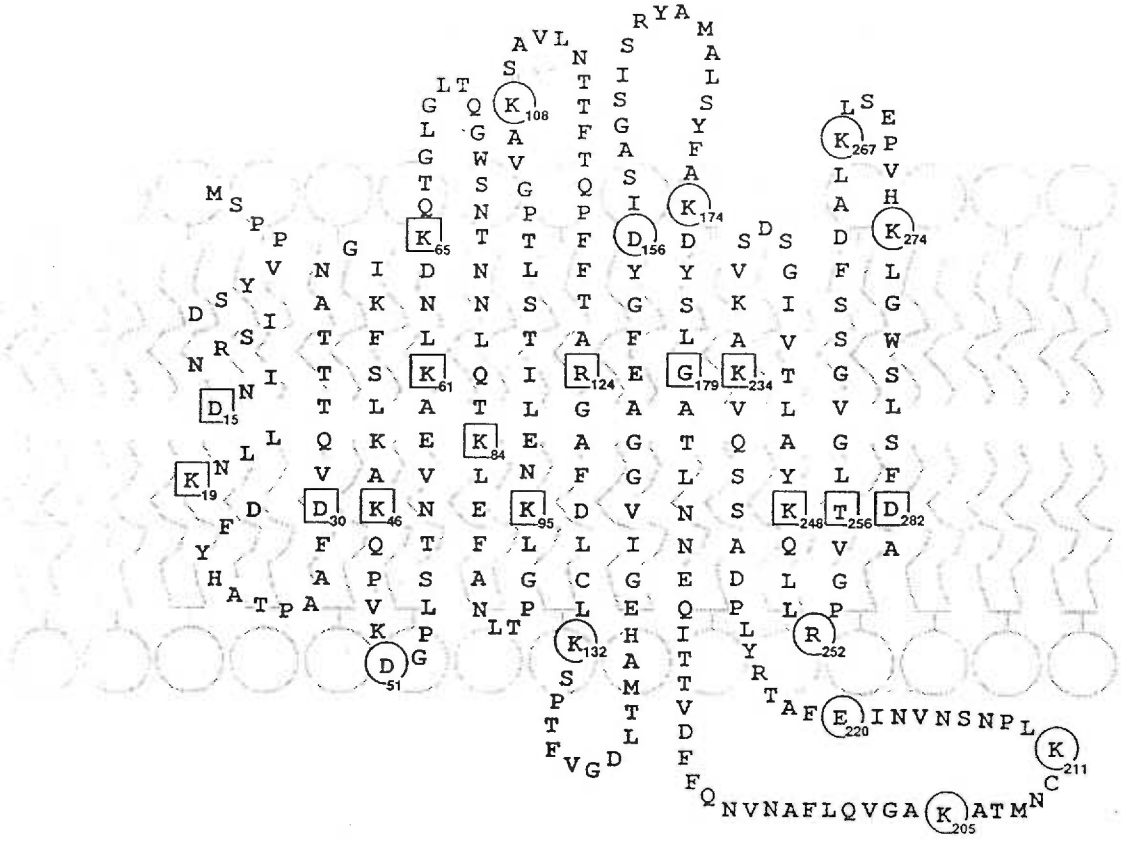
β sheet segments. In the yeast sequence, 12 major peaks of sided β structure and a few minor peaks were identified. The N terminal 20 amino acids that have been implicated as the sequence which targets VDAC to the outer mitochondrial membrane (Mihara and Sato 1985) can form an amphiphilic or sided α-helix (Kleene, Pfanner et al. 1987). VDAC from human, *Neurospora crassa* (Colombini, Peng et al. 1992) and rat (Bureau, Khrestchatisky et al. 1992) appear by this analysis to have the same pattern of sequence organization (Blachly-Dyson, Peng et al. 1989). Consistent with this idea, circular dichroism studies indicate that VDAC has an extended β-sheet conformation [for review, see (Mannella and Tedeschi 1992)].

Structure of the open state

A molecular model for the open state of yeast VDAC has been proposed based on functional studies of VDAC proteins containing site-directed mutations (Blachly-Dyson, Peng et al. 1990). This model was developed by changing the net charge at specific locations in the VDAC protein and determining the effect of the change on the selectivity of the resulting channels. Sites for mutation were selected either in proposed transmembrane strands (based on the analysis described above) or in intervening loops. Mutations which altered the selectivity of resulting channels were proposed to reside in the stream of ion flow (i.e., to line the channel) while those with no effect on selectivity were proposed to lie in intervening loop regions connecting transmembrane segments. In most cases, the observed results agreed well with the 12-β-strand-plus-α-helix model developed by analysis of structural motifs present in VDAC primary amino acid sequences. In this model, each polypeptide is proposed to form an α-helix at its N-terminus and 12 transmembrane β strands (Fig. 1). These transmembrane domains form the wall of VDAC channel with hydrophobic

Figure 1. A schematic model of the VDAC molecule

A Schematic model of the transmembrane topology yeast VDAC molecule in the open channel configuration. An NH_2-terminal α helix (left) is flanked by 12 strands of antiparallel β sheet. Residues which altered selectivity when changed by site-directed mutagenesis are boxed; residues in which mutations left the selectivity unchanged are circled (Taken from Blachly-Dyson, Peng et al., 1990).



residues towards the lipid membrane and hydrophilic residues towards the aqueous pore.

The location of individual domains in the open VDAC channel has been assessed using antibodies generated to a variety of peptide sequences found in VDAC. Using antibodies generated to N-terminal domains, DePinto et al. found (De Pinto, Prezioso et al. 1991) that the amino-terminal region of rat VDAC was not embedded in the lipid bilayer. Instead, it was exposed to cytosol although this view has been questioned by Stanley et al. who generated peptide-specific antibodies to probe the topology of *Neurospora crassa* VDAC. These authors found that an antibody against residues 1-20 (amino terminus) binds more efficiently to fungal mitochondria after outer membrane lysis, indicating that the N-terminus is accessible only on the interior surface of the outer mitochondrial membrane (Stanley, Dias et al. Submitted).

Regulation of VDAC channel

The properties of VDAC channels can be dramatically modified by addition of a variety of external factors which may relate to the function of these channel *in vivo*. Channels can be modulated by micromolar quantities of aluminum (Dill, Holden et al. 1987; Zhang and Colombini 1989), negatively charged polyanion (Colombini, Yeung et al. 1987; Mannella and Guo 1990) and a evolutionarily conserved protein, the VDAC modulator (Holden and Colombini 1988; Liu and Colombini 1991).

VDAC is regulated by aluminum and polyanions

Dill et al. found that adding micromolar quantities of aluminum chloride to VDAC channels incorporated into planar bilayers greatly inhibits their voltage dependence (Dill, Holden et al. 1987). The active aluminum

species is $Al(OH)_3$ formed by the addition of $AlCl_3$ to aqueous solutions (Zhang and Colombini 1989). Aluminum-treated channels exhibit two characteristic changes when compared to untreated channels: 1). Higher membrane potentials are needed to switch channels to closed states. 2). The steepness of the voltage dependence decreased while the voltage needed to close half the channels increased. The biological function of this regulation by aluminum is unclear although aluminum is present in human blood at micromolar levels.

VDAC properties are also regulated by polyanions (Colombini, Yeung et al. 1987). For example, a synthetic polyanion composed of a 1:2:3 copolymer of methacrylate, maleate and styrene (König's polyanion) increases the voltage dependence of VDAC channels over 5-fold at nanomolar levels. Some channels were totally blocked while others display higher voltage dependence and were able to close at very low membrane potential. At higher concentrations of this polyanion (27 μg/ml), VDAC channels were closed while being inserted into liposomes even in the absence of applied potential. Since this polyanion contains a mixture of carboxyl groups and nonpolar groups, this polyanion is thought to bind to VDAC through the nonpolar residues (Colombini, Yeung et al. 1987). The polyanion also affects the ion selectivity of VDAC. When applied to liver VDAC reconstituted into bilayers, channel P_{cation}/P_{anion} ratios increase (from <1 to >1) as the concentration of polyanion increases consistent with a transition from anion selective to cation selective channels. Asymmetric addition of the polyanion changes channel gating only when the polarity of the transmembrane voltage is negative on the cis-side (the side of the addition of the polyanion). If the voltage is positive on the cis-side, single channel conductance and selectivity of the channel are not affected by polyanion addition (Benz, Kottke et al.

1990). This action of König's polyanion is mimicked by other synthetic polyanions such as dextran sulfate (Mangan and Colombini 1987).

The VDAC modulator

Mitochondria contain a protein which has been termed the VDAC modulator which specifically changes VDAC's properties (Holden and Colombini 1988). VDAC modulator was first discovered in *Neurospora crassa* (Holden and Colombini 1988) and then found in the mitochondria of other eukaryotes such as rat and potato (Liu and Colombini 1991). The modulator is a soluble protein found in the intermitochondrial membrane space. In semi-purified preparations, this protein enhances the sensitivity of VDAC to voltage in three ways: increasing the rate of channel closure; decreasing the rate of opening and inducing channels to enter the closed state. However, modulator-enhanced closure requires that the applied potential be negative on the side of addition. The function of this protein appears highly conserved; modulator from one species increased the closing rate of VDAC channels from three different species. Further studies indicated that modulator increases the voltage dependence of VDAC channels over 2-3 fold. At higher concentration of VDAC modulator, some channels seem to remain in a closed state or are blocked while others display the higher voltage dependence and are able to close at low membrane potentials (Liu and Colombini 1992).

Physiological functions of VDAC

VDAC is the hexokinase and glycerol kinase binding site of mitochondria

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, Benz et al. 1982; Lindén, Gellerfors et al. 1982; Nakashima, Mangan et al. 1986). Binding may occur specifically at the contact sites between the inner and outer mitochondrial membrane (Kottke, Adam et al. 1988; Brdiczka and Wallimann 1994). Four isoforms of hexokinase are expressed in mammalian cells. The HK1 isoform appears to be specifically associated with mitochondria. Electron microscopic studies using polyclonal anti-hexokinase I antibodies have demonstrated that bound hexokinase is localized at the surface of brain mitochondria; a significantly higher concentration of gold grains was present at surfaces of the outer membrane attached to the inner membrane as opposed to outer membrane surfaces separated from the inner membrane (Kottke, Adam et al. 1988). Digitonin disruption of the outer mitochondrial membrane did not remove bound hexokinase from brain mitochondria although the activity of outer membrane markers and adenylate kinase decreased, consistent with the electron microscopy studies showing that hexokinase I is localized at contact sites (Kottke, Adam et al. 1988). Brdiczka and Wallimann also found that after digitonin treatment of liver mitochondria, labeled hexokinase was found in outer membrane vesicles that remained attached to the inner membrane (Brdiczka and Wallimann 1994). The binding of enzymes to the contact sites of mitochondria links cytoplasmic metabolism and ADP production with the regulation of mitochondrial respiration in the mitochondrial matrix (Polaskis and Wilson 1985; Kottke, Adam 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, Griffin et al. 1991)]. This

view has recently been questioned however (Kabir and Nelson 1991). Kabir and Nelson isolated mitochondria from rat brain and measured the activity of bound hexokinase in the presence of ADP and succinate or in the presence of added ATP. They found that external ATP supports a 2-3 fold higher hexokinase activity than does ATP generated by oxidative phosphorylation.

Results from several laboratories indicate that the mitochondrial receptor for hexokinase is indistinguishable from VDAC. 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, Gellerfors et al. 1982). VDAC is the major mitochondrial protein which binds to [^{14}C] dicyclohexylcarbodiimide (DCCD) at relatively low dosage (2 nmol 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 nmol of DCCD/mg of mitochondrial protein (Nakashima, Mangan et al. 1986). Evidence directly demonstrating that VDAC and the hexokinase binding protein are the same comes from the work of Fiek et al. in which the hexokinase binding protein and mitochondrial VDAC were isolated from rat liver mitochondria by different procedures. Hexokinase-binding protein made lipid vesicles permeable to ADP and formed asymmetric pores in lipid bilayer membranes identical to those obtained from VDAC. In addition, both hexokinase-binding protein and VDAC have the ability to bind glycerol kinase (Fiek, Benz et al. 1982).

The binding of hexokinase and glycerol kinase to mitochondria is dynamic, varying in tissues, developmental stage and metabolic state [for review, see (Adams, Griffin 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 was mitochondrial bound [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, Woods et al. 1967; Weinhouse 1972; Singh, Singh et al. 1974; Bustamante and Pedersen 1977; Bustamante, Morris et al. 1981; Nakashima, Paggi et al. 1984). In normal liver cells, particulate hexokinase distributes with nonmitochondrial membrane markers while particulate hexokinase distributes with outer mitochondrial membrane markers in transformed cells. Therefore, transformed cells seem to possess a higher percentage of mitochondrially bound hexokinase compared to the normal cells (Nakashima, Mangan et al. 1986). Direct demonstration of this notion has been obtained in the rodent tumor cell line, AS-30D. In these cells, mitochondrially bound hexokinase activity is three-fold elevated when compared to brain, the tissue with the highest total hexokinase activity (Arora and Pedersen 1988). It has been suggested that the increase in hexokinase activity bound to the outer mitochondrial membrane observed in tumor cells may be responsible for the increased glycolytic rate observed as a characteristic of these cells, even when they are in the presence of adequate oxygen (Adams, Griffin et al. 1991).

VDAC is a part of mitochondrial benzodiazepine receptor complex

VDAC appear also to form a component of the mitochondrial benzodiazepine receptor complex (McEnery, Snowman et al. 1992). Benzodiazepines are effective anticonvulsants, antianxiety drugs which appear to bind to two kinds of receptors: central receptors and peripheral receptors. cDNAs encoding the central benzodiazepine receptor have been

cloned, expressed in frog oocytes (Schofield, Darlison et al. 1987) and identified as a macromolecular complex which serves as the receptor for the inhibitory neurotransmitter GABA, consistent with the evidence that benzodiazepines exert their CNS effects by facilitating the synaptic effects of GABA.

The peripheral benzodiazepine receptor (PBR) is a distinct receptor which is similar to the central receptor in its affinity for diazepam but differs in its affinity for other drugs. For instance, clonazepam, one of the most pharmacologically potent benzodiazepines, has a K_i value for CNS binding sites in the low nanomolar range, but is several thousand-fold weaker at peripheral sites. PBR is expressed at high levels in the adrenal gland and a variety of non-neuronal glandular tissues including the salivary gland, the testis and the ovary. Other nonglandular tissues, such as the nasal epithelium, lung and kidney, also have high levels of PBR. Benzodiazepines appear to affect many biological functions through PBR. Benzodiazepine alter both the release of growth hormone, ACTH, prolactin, and luteinizing hormone from anterior pituitary gland and affect plasma levels of adrenocortical and testicular hormones (Marc and Morselli 1969; Anderson, Bankier et al. 1981). Diazepam inhibits aldosterone production in bovine adrenal glomerulosa cells (Shibata, Kojima et al. 1986), indicating a direct action of benzodiazepines on adrenal function as mediated by PBR. The effects of peripherally-selective benzodiazepines at micromolar concentration include inhibition of the *in vitro* growth of thymoma cells (Wang 1984), depression of cardiac muscle contractility (Mestre, Carriot et al. 1984) and induction of melanoma cells (Matthew, Laskin et al. 1981).

Subcellular fractionation studies indicated that PBR is localized in mitochondria (Anholt, Pedersen et al. 1986). Relative densities of peripheral

receptors in various subcellular fractions correlate closely with a mitochondrial marker, cytochrome oxidase but not with markers for other cellular organelles. Further fractionation of mitochondria suggest that the PBR is specifically localized in the outer mitochondrial membrane. Biochemical purification of the PBR indicates that the PBR appears to be composed of a complex of distinct 18 kDa, 30 and 35 kDa protein subunits. The 18 kDa protein is labeled by the isoquinoline carboxamide ligand ^{3}H-PK 14105 (Dobel, Ferris et al. 1987; Beyer, Hirsch et al. 1988; Skowronski, Fannestil et al. 1988) and has been cloned (Sprengel, Werner et al. 1989). Thirty kilodalton and 35 kD proteins are labeled with ^{3}H-flunitrazepam (Paul, Kempner et al. 1981). Selective antibodies and reagents identified the 30 kD protein as VDAC and the 35 kD protein as the adenine nucleotide carrier (ADC), an inner mitochondrial membrane transporter. VDAC and ADC together with the 18 kDa protein appear then to form a complex that constitutes most of the PBR binding activity (McEnery, Snowman et al. 1992).

Multiple mammalian VDAC isoforms

Several lines of evidence suggested that more than one VDAC gene is expressed in mammals. First, electrophysiological analysis has demonstrated that the membrane conductance induced by rat brain VDAC appeared to be less voltage-dependent than that of VDAC purified from rat liver (Ludwig, De Pinto et al. 1986). The number of charges involved in the gating process of rat brain VDAC was approximately one but three for rat liver VDAC. This difference is independent of the lipid composition of the membrane, suggesting to these authors that there may be a difference either in the sequence or in the complex forming each VDAC channel. Second, Dorbani et al. (1987) fractionated rat brain nonsynaptic mitochondria by digitonin. Two

populations of VDAC were identified; one binding most of the hexokinase and associated with the inner membrane and another which did not bind hexokinase and appeared to be associated with outer membranes not in contact with inner membranes (Dorbani, Jancsik et al. 1987) suggesting that two populations of functionally distinct VDAC molecules may exist. Third, 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. The N-terminal 15 amino acids of rat HK1 are necessary and sufficient to confer mitochondrial binding (Gelb, Adams et al. 1992). The N-terminal 15 amino acids of HK1 isolated from c37 mouse hepatoma cell line is identical to that of HK1 associated with non-mitochondrial binding sites in normal liver cells (Schwab and Wilson 1989; Arora, Fanciulli et al. 1990). These results suggest that the mitochondrial binding domain of HK1 is the same in both transformed and normal cell lines and further, that the redistribution of hexokinase from nonmitochondrial compartments in normal cells to mitochondria in transformed cells is due to the association with different populations of receptors. Thus, it is possible that there may be multiple forms of VDAC in mammals that differ in their localization in cells and in physiological functions.

Evidence for the localization of VDAC in other cell compartments

Although VDAC protein has been found exclusively in mitochondria from all species examined, a number of reports suggested that VDAC-like channel activity may also exist in cell compartments other than the mitochondria.

Evidence from electrophysiological studies

Electrophysiological experiments indicate that VDAC-like channels are present in the plasma membrane (Blatz and Magleby 1983; Jalonen, Johansson et al. 1989; Dermietzel, Hwang et al. 1994). Blatz and Magleby used single channel, patch-clamping techniques to record from 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 is 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 VDAC channel at this salt concentration (0.4-0.5 nS in 0.1 M KCl) (Freitag, Neupert et al. 1982; De Pinto, Benz et al. 1989; De Pinto, Prezioso et al. 1991). In addition, the channel is active at 0 mV and inactive at negative or positive membrane potentials, again similar to the properties of mitochondrial VDAC channel. This channel was observed however, 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, Johansson et al. 1989). Kolb et al. also 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)].

Evidence from immunocytochemical studies

Polyclonal antibodies have been raised against the complete human VDAC molecule purified from human B lymphocytes (porin 31HL) and against a synthetic peptide corresponding to 19 amino acids found at the N-terminus of this protein. This protein has been subsequently shown to be identical to HVDAC1. Antibodies were used in indirect immunofluorescence studies of both living and fixed H2LCL cells and each shown to specifically

label the plasma membrane of these cells (Thinnes, Götz et al. 1989). In addition, eight monoclonal antibodies against porin 31HL have also been described by Babel et al.(1991). All of the eight monoclonal antibodies reacted equally with peptides representing the N-terminus of HVDAC1 and four stained the plasma membrane of human skeletal muscle (Babel, Walter 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, Götz et al. 1991). EBV-transformed human B lymphocytes showed positive plasma membrane staining, as did several normal human B and T cell lines (König, Götz et al. 1991). These results suggest that the presence of porin 31HL on the plasma membrane is not the result of cell transformation.

In addition to the light level immunocytochemical studies, these monoclonal antibodies were also used at the electron microscopic level to demonstrate the presence of porin 31HL in the plasma membrane (Cole, Adil Awna 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, porin 31HL appeared to be present in the plasma membrane. In all of these studies, the authors report no mitochondrial labeling although recent immunoelectron microscopy studies indicated that these antibodies also label isolated rat heart mitochondria (Konstantinova, Mannella et al. 1995).

Studies in Astrocytes

Dermietzel et al. purified a VDAC-like protein from bovine brain. A cDNA clone encoding this protein was obtained from a bovine brain cDNA library using probes generated from the amino acid sequences of the purified

protein. The derived amino acid sequence demonstrates that this clone encodes the bovine homolog of HVDAC1 or porin 31HL (BR1-VDAC). Immunolocalization at both the light and E.M. level using one of the eight monoclonal antibodies recognizing the N-terminal 19 amino acids of porin 31HL indicated that the protein encoded by this cDNA is specifically associated with the plasma membrane of astrocytes in rat brain. Furthermore, VDAC-like channel activity was recorded by patch clamp recording of the astrocyte plasma membrane. This channel has single channel conductance of 434 pS in 143 mM KCl and is anion selective, similar to that observed for mitochondrial VDAC following reconstitution into planar bilayers. Addition of the same antibody used in immunolocalization studies to the incubation buffer blocked channel activity (Dermietzel, Hwang et al. 1994). Together, these results suggest the presence of porin 31HL (or HVDAC1) in the plasma membrane of rat astrocytes.

Co-purification of VDAC with proteins normally found in plasma membrane.

Rat VDAC has been co-purified with the γ-aminobutyric acid type A ($GABA_A$) - Benzodiazepine receptor on a benzodiazepine affinity columns (Bureau, Khrestchatisky et al. 1992). The $GABA_A$ receptor from mammalian brain is a hetero-oligomer consisting of four or five subunits. Subunits α, β, γ, δ have been cloned, each of which exists as a family of several subtypes (Olsen and Tobin 1990; Sigel, Baur et al. 1990; Verdoorn, Draguhn et al. 1990). Bureau et al. 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. 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, although there are differences in the sequence of the protein encoded by this cDNA and the peptide sequence generated from the purified protein. The purified 36 kDa polypeptide forms a channel in lipid bilayer membranes with properties identical to that of VDAC isolated from mitochondria of various sources. An antiserum raised against purified 36 kDa polypeptide was able to adsorb significant amounts of purified $GABA_A$ receptor protein as assayed by [^{3}H] muscimol binding (Bureau, Khrestchatisky et al. 1992); 60% of the binding activity is absorbed when undiluted antisera was used and 30% absorbed with a 1:10 dilution of this antisera.

VDAC has also been copurified with plasma membrane specializations such as caveolae (Lisanti, Scherer et al. 1994). Caveolae are 50-100 nm membrane domains representing a sub-compartment of the plasma membrane. Caveolin is a 22 kDa integral membrane protein which is an important structural component of caveolae. Lisanti et al. developed a procedure for isolating caveolin-rich membrane domains from cultured cells. By microsequencing, they found that VDAC appears to be present in this caveolin-rich membrane domain (Lisanti, Scherer et al. 1994).

Objectives and approaches

As indicated above, VDAC appears to mediate a variety of biochemical interactions and functions. Consistent with the many processes attributed to this molecule, available evidence suggests that multiple isoforms of VDAC exist in mammalian cells. Since cDNAs encoding one human VDAC isoform (HVDAC1 or porin 31HL) had been identified, one of the goals of the work described in this thesis was to identify and characterize cDNAs encoding additional mammalian VDAC isoforms. A variety of studies also suggest that

VDAC can exist in cellular compartments in addition to mitochondria. Thus, a second goal of this thesis work was to determine the subcellular location of individual VDAC isoforms in mammalian cells.

In order to identify additional human VDAC isoforms, a probe derived from the HVDAC1 cDNA was used to screen a human liver cDNA library at both low and high stringency. Clones hybridizing at low but not high stringency were identified and characterized further. This characterization, as well as work by others, indicated that the various VDAC isoforms expressed in human cells differ primarily by the presence or absence of N terminal extensions. Thus, these differences in the N termini of human VDAC isoforms raised the possibility that each isoform may be targeted to different cell compartments. In order to test the hypothesis that members of the VDAC family may be located in cellular compartments other than the mitochondrial outer membrane, we took advantage of the availability of antibodies which specifically recognize two distinct peptide sequences. cDNAs encoding individual human VDAC isoforms were then modified so that the encoded proteins contain C terminal sequences recognized by antibodies to either of these epitopes. These epitope tags appeared to have no effect on the function of modified VDAC proteins. Epitope-tagged HVDAC isoforms were then individually expressed in COS7 cells or rat astrocytes by transient transfection. Subcellular fractionation, light level immunocytochemistry and immunoelectron microscopy was subsequently used to identify the subcellular localization of each epitope-tagged HVDAC isoform. In addition, since multiple VDAC genes are expressed in the same cell, it is possible that more than one human VDAC isoforms is present in the same cell compartment. Thus, individual cell compartments may exclusively contain one VDAC isoform or multiple isoforms may be present in the

same compartment. To distinguish these alternatives, constructs encoding human VDAC isoforms containing different epitopes were expressed in the same cultured mammalian cell. The subcellular location of each isoform was then assessed by both light level immunocytochemistry and immunoelectron microscopy.

Material and Methods

Antibodies and Cell Lines

A mouse monoclonal antibody (M2) recognizing the FLAG epitope was purchased from International Biotechnologies (New Haven, CT), and rabbit polyclonal antibodies recognizing this epitope were kind gifts of Dr. Gary Ciment, Oregon Health Sciences University. A mouse monoclonal antibody recognizing the HA (12CA5) epitope was purchased from Boehringer-Mannheim (Indianapolis, IN), and rabbit polyclonal antibodies (PRB-101C) recognizing this epitope were purchased from BabCo (Richmond CA). Fluorescein and rhodamine conjugated secondary antibodies and avidin were purchased from Vector Laboratories (Burlingame, CA). Gold-conjugated secondary antibodies were purchased from Amersham International Inc. (Chicago, IL). Polyclonal antibodies to mammalian cytochrome C oxidase holoenzyme and the monoclonal antibody against cytochrome C oxidase subunit IV (10G8-D12-C12) were kind gifts from Dr. Jan-Willem Taanman (University of Oregon, Eugene, OR). Polyclonal antibody against Signal Sequence Receptor was provided by Dr. Rapopport (Berlin, GDR) (Prehn, Herz et al. 1990).

Rat astrocytes were a kind gift of Dr. Felix Eckenstein (Oregon Health Sciences University, Portland, OR).

Isolation and sequencing of HVDAC2

A human liver λ gt 11 cDNA library (kindly provided by Dr. N. Kennaway, Oregon Health Sciences University) was screened by a 750 bp HindIII fragment containing most of the coding region of HVDAC1 (Blachly-Dyson, Zambronicz et al. 1993). Duplicate filters were probed and washed

either at high stringency or at low stringency. Hybridization at high stringency was carried out at 42° C in hybridization buffer [5 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate), 0.2 % SDS, 5 x Denhart's, 50 mM sodium phosphate, pH 7.2, 1% milk, 0.1% sodium azide] containing 50% formamide and filters were washed in 0.1 x SSC, 0.2% SDS at 65° C. Hybridization at low stringency was carried out at 37°C in hybridization buffer containing 30% formamide and filters were washed in 1 x SSC, 0.2% SDS at 50°C.

DNA probes used for library screening were labeled with α-^{32}P dCTP (NEN Dupon) by nick translation (BRL kit) or random priming method (Boehringer Mannheim random priming kit) as described (Maniatis, Fritsch et al. 1982). Plasmid DNA isolation was carried out using alkaline lysis procedure (Maniatis, Fritsch et al. 1982). Phage DNA isolation was carried out by the glycerol gradient procedure (Maniatis, Fritsch et al. 1982).

DNA sequencing

Fragments were subcloned into Bluescript vector (Stratagene). Sequencing reactions were carried out using the dideoxynucleotide method (Sanger, Nicklen et al. 1977) with Sequenase (United States Biochemical) and ^{35}S dATP. T3 and T7 sequencing primers were used and the reactions run on 5% denaturing polyacrylamide gels.

Epitope-tagged cDNA constructs

For HVDAC1, fragments were generated by polymerase chain reaction (PCR) using oligonucleotides spanning an internal EcoRI site and inserting a novel ClaI site at the stop codon. For HVDAC2 and HVDAC2', fragments were generated by PCR using oligonucleotides spanning an internal BglII site and inserting a novel ClaI site at the stop codon. These fragments were then

used to replace the corresponding fragments in each cDNA. Double stranded DNA cassettes encoding the FLAG (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) and HA (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala) epitopes containing 5' ClaI sites and 3' XhoI sites followed by a stop codon were inserted between the newly created ClaI sites at the C terminus of each gene and a vector XhoI site to create cDNAs encoding HVDAC1, HVDAC2 and HVDAC2' epitope containing molecules (Fig. 2).

Expression of epitope-tagged HVDACs in yeast

The HVDAC-FLAG and HVDAC-HA constructs were inserted into the multicloning sites of a yeast expression vector pSEYC58 (Emr, Vassarotti et al. 1986). The pSEYC58 plasmid contains a yeast URA3 gene, ARS1 (autonomous replication site) and CEN4 (centromere from chromosome IV) inserted into pBR322.

Epitope-tagged HVDAC was expressed in yeast by transformation. Yeast transformation was performed following a modified method published by Schiestl (Schiestl and Gietz 1989). A colony of m22-2 strain, a yeast strain lacking the endogenous yeast VDAC gene (Blachly-Dyson, Peng et al. 1990), was inoculated in 10 ml of YPD medium (Bio 101, Inc., La Jolla, CA) and incubated overnight at 30° C. Yeast cells were then diluted to an OD_{600} of 0.5 in 50 mls of YPD medium and grown for an additional four hours. Following this incubation, cells were harvested by centrifugation, washed with 40 mls of 10 mM Tris, 1 mM EDTA, pH 8.0 (TE), re-pelleted, resuspended in 2 mls 100 mM lithium acetate containing 0.5 x TE and then incubated at room temperature for 10 min. One hundred microliters of the yeast suspension was added to a 1.5 ml tube containing a mixture of 1μg of plasmid

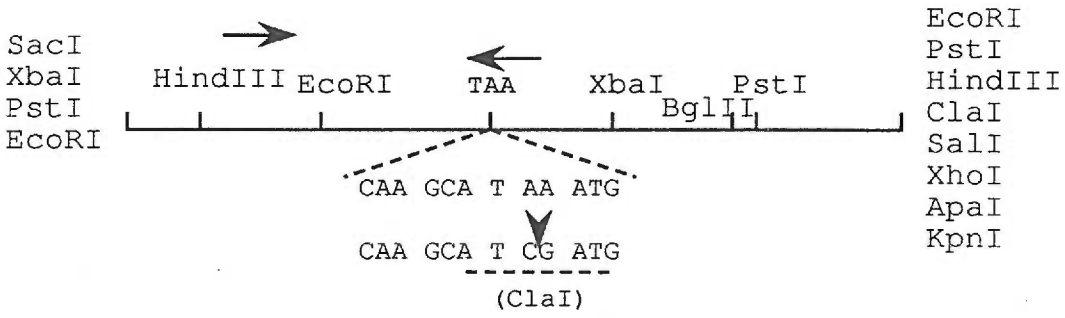
Figure 2. Schematic outlining the construction of HVDAC1-FLAG constructs

A. A fragment was generated by PCR from HVDAC1 cDNAs using an oligonucleotide spanning a 5' internal EcoRI site and a 3' oligonucleotide inserting a novel ClaI site at the stop codon. This fragment was used to replace the corresponding fragment in the HVDAC1 cDNA. Line: HVDAC1cDNA; arrow: PCR primer.

B. Double stranded DNA cassette encoding the FLAG epitope containing a 5' ClaI site and a 3' XhoI site followed by a stop codon was inserted between the newly created ClaI site at the C-terminus of HVDAC1 and a vector XhoI site.

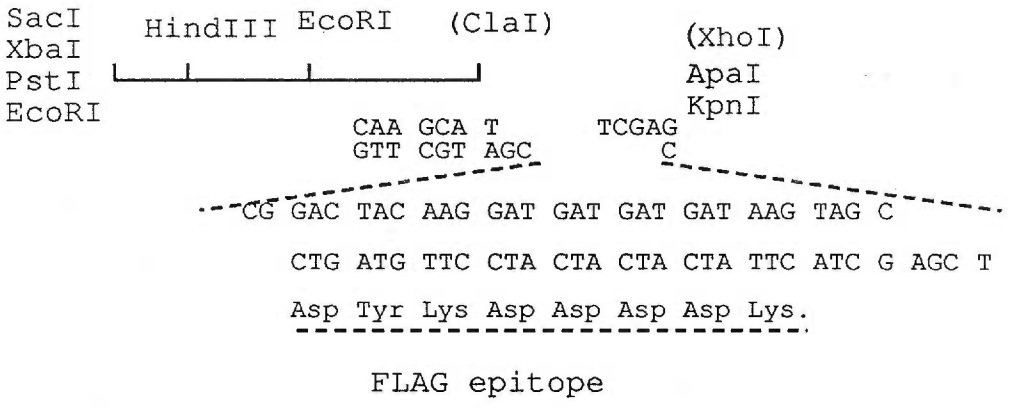
A.

HVDAC1cDNA



B.

HVDAC1-FLAG



DNA and 100 μg denatured and sheared salmon sperm DNA. The yeast cells were mixed well with DNA by vortexing. Seven hundred microliters of 100 mM lithium acetate/40% PEG (Polyethylene glycol) 3350/1 x TE were added to the mixture, vortexed vigorously and incubated at 30° C for 30 minutes. Following this incubation, 88 μls of DMSO was added to the mixture and the cells were then shocked by heating to 42° C for 7 minutes. The heat-shocked yeast cells were washed with 1.0 ml of water, resuspended in 50-100 μls of TE and then plated on SD-ura selective media. Colonies were picked after three days. One liter SD-ura medium contains 44 gm of DOBA (Dropout agar base for synthetic defined drop-out media, Bio 101, Inc., La Jolla, CA) and 20 mls of 50 x (-ura). 50 x (-ura) = 0.15% Adenine, 0.1% Histidine, 0.15% Lysine, 0.15% Leucine and 0.12% Tryptophan (w/v).

Expression of epitope-tagged HVDACs in mammalian cells

For the expression of epitope-tagged HVDACs in COS7 cells, the HVDAC-FLAG and HVDAC-HA constructs were inserted into the multicloning sites of pCD-PS (Bonner, Young et al. 1988) and pRC/RSV (Invitrogen, San Diego, CA) mammalian expression vectors. For the expression of HVDAC-FLAG in astrocytes, a modified pcDNA3 (Invitrogen, San Diego, CA) kindly provided by Dr. John Adelman (Vollum Institute) was used.

COS7 cells, Ltk^- cells and rat astrocytes were cultured in monolayer in Dulbecco's modified Eagle's medium (DMEM) containing 10% of heat inactivated fetal calf serum in atmosphere of 5% CO_2 at 37° C.

Mammalian cell transfection

Transient transfection

For transient expression of epitope-tagged HVDAC molecules in COS7 cells and astrocytes, cells were transfected using a protocol modified from Chen and Okayama (Chen and Okayama 1987). COS7 cells, maintained in DMEM supplemented with 10% of fetal calf serum , were seeded at 2-5 x 10^6 cells per 100 mm plate and grown overnight. The cells were refed with fresh medium 3-4 hours prior to transfection. Twenty micrograms of plasmid DNA was mixed with 0.5 ml of 0.25M $CaCl_2$ in a 15 ml sterile tube. One half milliliters of 2 x BBS [50mM N-,N-Bis(2-hydroxyethyl)-2-amino-ethanesulfonic acid (Calbiochem), 280 mM NaCl, 1.5 mM Na_2HPO_4 , pH 6.95, adjusted with NaOH] was added dropwise to the tube. The tube was capped and incubated at room temperature for 15-30 minutes until a precipitate was visible under the microscope. The precipitate was then added to the cell plate drop wise and mixed gently by tipping the plate back and forth. The cells were incubated for overnight, washed with PBS three times, and incubated for an additional 24 hours before fixation and harvesting.

Stable transfection

For stable expression of epitope-tagged HVDAC in Ltk^- cells, cells were transfected using a protocol modified from Chen and Okayama (Chen and Okayama 1987). Ltk^- cells were seeded at 30% confluence in 100 mm plates. Cells were transfected the following day by the same method as the transient transfection. HVDAC-FLAG cDNA constructed in a mammalian expression vector containing neo^r cassette was used to transfect Ltk^- cells. 48 - 72 hours after transfection, each 100 mm plate of cells was split into four 150 mm plates. The cells were grown for additional 10 days in DMEM containing penicillin/streptomycin (pen/step, 50 unit/50 μg per ml) and G418 (700

μg/ml). Individual colonies were identified, plated in 24 well plates, and cultured in media with G418 and pen/strep.

Mammalian DNA isolation

DNA was isolated from cultured mammalian Ltk$^-$ cells using the procedure described by Laird, P.W. et al. (Laird, Zijderveld et al. 1991).

The cells were treated with trypsin, harvested by centrifugation and incubated in 0.5 ml of lysis buffer (100 mM Tris-HCl pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl and 100 μg/ml proteinase K) for 2 hours at 37° C with shaking. One volume of isopropanol was added to the lysate and the samples mixed until precipitation was complete. DNA was recovered by lifting the aggregated precipitate from the solution, the precipitate rinsed twice with 75% ethanol and air dried. The dried DNA was then dissolved in 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5.

PCR analysis of HVDAC-FLAG transfected Ltk$^-$ cells

DNA isolated from HVDAC-FLAG transfected Ltk$^-$ cells was analyzed by PCR. Twenty five microliter PCR reactions contained 40 μM dNTPs, 1.25 U Taq polymerase (Boehringer Mannheim), Taq polymerase buffer and 5 pmols of the forward and reverse primers. The sequences of these oligomers correspond to residues 721-745 of HVDAC2 (R873), antisense sequence corresponding to residues 1093-1114 of HVDAC2 (R874), and antisense sequence of FLAG (S529) respectively.

R873: GCT TGG ACA TCA GGT ACC AAC TGG

R874: AAC CAG CTA ACA AAG AAC TGT C

S529: TTA TCG TCA TCG TCT TTG TAG TCT

The PCR was carried out for 40 cycles, each consisting of denaturation at 96° C for 30 sec., annealing at 60° C for 30 sec., and extension at 72° C for 90 sec. The samples were electrophoresesed on a 1% agarose gel and viewed under UV light.

Yeast mitochondria isolation

Yeast mitochondria were isolated from m22-2 and strains expressing epitope-tagged HVDACs using a modified procedure described by Daum and colleagues and Blachly-Dyson and colleagues (Daum, Böhni et al. 1982; Blachly-Dyson, Peng et al. 1990). Yeast cells grown to late log phase were harvested by centrifugation and incubated in 0.1M Tris pH 9.5, 0.01M dithiothreitol for 10 min. at 30° C. Cells were then washed in 1.5 M sorbitol, 0.01 M Tris pH 7.5 and incubated with zymolyase at 3 mg/gm of cells at 30° C for 60 min. in the same solution. Cells were disrupted at medium speed in glass/teflon homogenizer in 0.3M sucrose, 10mM Tris-HCl, pH 7.5, 0.3% bovine serum albumin, and mitochondrial pellets were washed several times in the same buffer. Isolated mitochondria were lysed in 1 mM KCl, 1 mM Tris-HCl, pH 7.5, and the membranes collected by centrifugation at 27,000 x g for 20 min. Mitochondrial membranes were resuspended in 1mM KCl, 1 mM Tris-HCl and frozen in liquid N_2 for further use.

Preparation of total protein extracts

One hundred millimeter plate cell cultures were washed with 5 ml cold PBS three times followed by incubation in RIPA buffer (0.01M Tris-HCl pH 7.6, 0.15M NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 1% Aprotinin and 2mM sodium vanadate) on ice. Cellular material was harvested by scraping the plates and further incubation on ice for 10 minutes.

Samples were centrifuged at 12,000 x g for 10 minutes at 4° C in a microcentrifuge and supernatants mixed with SDS sample buffer. Phenylmethyl-sulfonyl fluoride (PMSF) was added to the sample prior to the centrifugation (2 mM final concentration). The protein concentration of the supernatant was determined by the method of Bradford (Simpson and Sonne 1982; Weiner and Kaminski 1990).

Subcellular fractionation

Subcellular fractions of transfected cells were prepared by first washing cells with ice cold PBS/0.1% glucose followed by incubation in 10 mM TrisCl pH 7.4, 10mM NaCl and 2mM $MgCl_2$ for 10 minutes on ice. Cells were then harvested by scraping, collected by centrifugation, homogenized with a Dounce tissue grinder and spun at 600 x g for 5 minutes at 4° C. PMSF (2 mM) was added prior to centrifugation. The resulting supernatant was centrifuged at 20,000 x g for 10 minutes to produce a low speed pellet and supernatant. The low speed supernatant was subjected to a subsequent centrifugation at 235,000 x g for one hour. The low speed and the high speed pellets were resuspended in the buffer used for washing cells containing PMSF. The protein concentration of various fractions was determined by the method of Bradford (Simpson and Sonne 1982; Weiner and Kaminski 1990).

Western blots

Cell extracts and fractions prepared as described above were separated by electrophoresis on 10% SDS-polyacrylamide gels. The proteins were transferred onto nitrocellulose (Costar) by electroblotting in transfer buffer (25 mM Tris, 192 mM Glycine, 20% Methanol and 50 mM NaCl, pH 8.3). Proteins were visualized with Ponceau S. The membrane was blocked in 5% nonfat

dry milk (Carnation Inc.) in TBST (0.15 M NaCl, 0.01 M Tris pH 8.0 and 0.1% Tween 20) for one hour. Primary antibodies were added and, after one hour incubation, membranes washed five times for 5 minutes in TBST. HRP-conjugated anti mouse or anti rabbit IgG (Santa Cruz Biotechnology), depending on the source of primary antibody, was incubated with the membrane at 1: 5000 in TBST with 5% of nonfat dry milk for 30-60 minutes. After washing 5 times for 5 minutes with TBST, the membrane was incubated with a mixture containing equal volumes of detection reagent 1 and detection reagent 2 (ECL, Amersham, Chicago, IL). Antibody binding was visualized by exposing blots to X-ray film in a dark room.

Immunocytochemistry

Light-level Immunofluorscence

COS7 cells and rat astrocytes were seeded onto glass coverslips and transfected as described above. Transfected cells were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde for 20 min at room temperature. Fixed cells were then washed with PBS containing 0.2 % BSA and 0.1% Triton X-100 (washing buffer) three times and incubated in PBS containing 10% horse serum and 0.1% Triton X-100 for one hour at room temperature. Primary antibodies (monoclonal or polyclonal) were diluted in washing buffer, applied to coverslips and placed at 4° C. Following overnight incubation, coverslips were washed five times with washing buffer and then incubated with FITC-conjugated anti-mouse or anti-rabbit antibodies depending on the source of primary antibody. For rhodamine detection, coverslips were incubated with biotin-conjugated anti-mouse or anti-rabbit antibodies depending on the source of primary antibody, then washed five

times with washing buffer, and finally incubated with avidin-rhodamine for 30 minutes followed by five washes.

Astrocytes were also processed for light level immunocytochemical analysis by the methods outlined in Dermitzel et al (Dermietzel, Hwang et al. 1994). Transfected cells were fixed in ethanol at -20° C for 20 min. and followed by PBS (phosphate-buffer saline) washing. Fixed cells were then incubated in PBS, supplemented with 0.1% albumin. Primary antibody was diluted in PBS, applied to the coverslips and placed at room temperature for one hour. Coverslips were washed three times with PBS and then incubated with FITC- or biotin-conjugated anti-mouse or anti rabbit antibodies depending on the source of primary antibody followed by five washes. For rhodamine detection, coverslips were incubated with avidin-rhodamine for 30 min after incubation with biotin-conjugated secondary antibody, followed by five washes.

Immunoelectron Microscopy

Transfected cells were washed once with Versene (0.53 mM EDTA in PBS) and incubated in 1 ml of Versene at room temperature for 3-5 min. The cells were lifted from the dish by tapping the culture dish and fixed with 2% paraformaldehyde, 0.1% glutaraldehyde in 50 mM Pipes (pH 7.2) for one hour on ice. Fixed cells were rinsed three times with 0.1 M Pipes, embedded in 2% agar, infiltrated with polyvinylpyrolidone and sucrose (20% polyvinylpyrolidone, 44 mM Na_2CO_3, 1.84 M Sucrose, 0.1 M Na_2HPO_4) overnight (Tokuyasu 1989), and prepared for frozen thin sectioning (Griffiths, Simons et al. 1983; Griffiths, McDowell et al. 1984). Frozen tissue was placed in liquid nitrogen and then sectioned. The sections are about 70 nm and were prepared with a Reichert ultra-cut E with an FC4 freezing attachment. Tissue sections were collected onto grids and stored in PBS containing 1% BSA. The

frozen sectioning was kindly carried out by Dr.Bill Wolfgang in our laboratory. The grids were incubated in 1% fishskin gelatin (made in PBS) for 10 minutes. Gelatin coated grids were then blocked in PBS containing 0.01M glycine and 10% fetal calf serum for 10 min. Primary antibodies were diluted in PBS containing 1% BSA and 0.01M glycine and applied to the grids. Grids were washed 4 times over 15 min. in PBS containing glycine and BSA. After incubating with primary monoclonal or polyclonal antibodies, anti-mouse or anti-rabbit secondary antibodies conjugated to gold particles (anti-mouse, 5 or 10 nm; anti-rabbit, 15 nm) were applied to the grids, and the grids were washed 5 times over 20 minutes in the washing buffer and 4 times over 15 min. in sterile water. Immunolabeled sections were stained with 0.3% uranyl acetate, embedded in methylcellulose (Griffiths, McDowell et al. 1984) and viewed under a JOEL JEM-100CXII transmission electron microscope.

Color Prints

Color negatives and slides were scanned with a Nikor LS-3500 Film Scanner. Images were reduced to appropriate layout sizes using the Adobe Photoshop 2.5 or 3.0 software at resolution of 300 to 600 pixels per inch and labeled with numbers and letters. No color adjustments were made. Some scanned files were placed into the Freehand 3.1 or 4.0 software and labeled with numbers and letters. Figures were then printed on a SuperMac Supermatch PTR 120 Proof Positive Dye Sublimation Printer at final resolution of 300 dots per inch.

Results

Identification of human VDAC genes

Identification of HVDAC1 cDNA

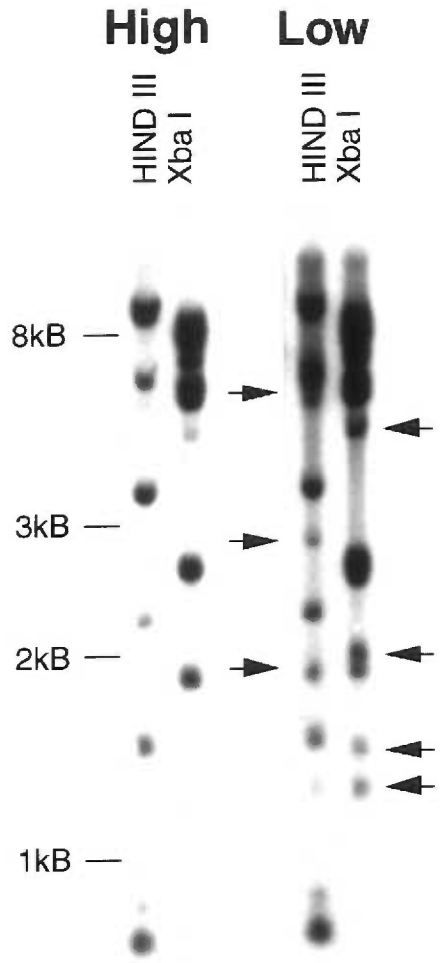
The first human VDAC protein, "Porin 31HL", was purified from the transformed human B-lymphocytes H2LCL cell line by Thinnes et al in 1989. The amino acid sequence of this protein was obtained by direct peptide sequencing (Thinnes, Götz et al. 1989). Based on this amino acid sequence information, cDNAs encoding this protein (HVDAC1) were identified in a human pituitary cDNA library. Initially two pairs of nested primers based on the sequence of "Porin 31HL" were used to amplify by PCR a 450 bp product from transcripts expressed in a human B-lymphocyte cell line. The sequence of this DNA fragment indicated the presence of an open reading frame encoding a polypeptide identical to amino acids 83-230 of "Porin 31HL". This fragment was then used to screen a human pituitary cDNA library. One full length cDNA clone, HVDAC1, was obtained and characterized. The insert contains an open reading frame encoding the primary sequence of "Porin 31HL" (Blachly-Dyson, Zambronicz et al. 1993).

Identification of HVDAC2 cDNA

Electrophysiological and biochemical evidence suggests the presence of more than one VDAC isoform. Initial genomic Southern-blots using probes generated from the HVDAC1 cDNA confirmed the existence of genes encoding additional human VDAC isoforms. Human peripheral blood genomic DNA was isolated and digested with either HindIII or XbaI and Southern blots probed with sequences representing the coding region of the

Figure 3. Genomic Southern-blot demonstrating the existence of additional HVDAC genes

Human genomic DNA from peripheral blood was isolated and digested with either HindIII or XbaI. Blots were then incubated with [^{32}P] labeled HVDAC1 cDNA at both high and low stringency and subsequently subjected to autoradiography. Arrows indicate the extra bands hybridizing at the low stringency compared to high stringency.


High
Low
HIND III
Xba I
HIND III
Xba I
8kB
3kB
2kB
1kB

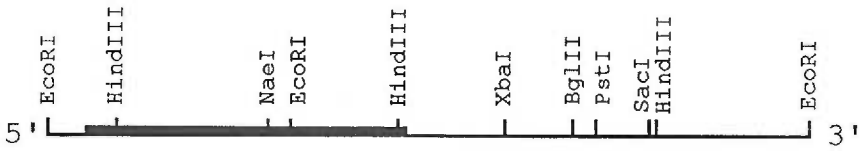
HVDAC1 cDNA at both high and low stringency. The HindIII digested sample showed at least three additional bands at the low stringency compared to high stringency, while the XbaI digested DNA sample had at least four more bands at low stringency compared to high stringency (Fig. 3).

In order to identify cDNAs encoding additional human VDAC isoforms, a 750-bp HindIII fragment of HVDAC1 containing most of the coding region was used to probe a human liver cDNA library at both high and low stringency. Five clones that hybridized at low, but not high stringency were identified and plaque purified. cDNA inserts contained in these phages were excised with EcoR1, subcloned into the plasmid Bluescript, subjected to restriction analysis and partially sequenced. Sequence analysis indicated that this set of phages represented overlapping clones encoding the same protein but differ primarily in extent of 5' sequence. The longest cDNA insert was completely sequenced and termed HVDAC2. Restriction maps of HVDAC1 and HVDAC2 are shown in Figure 4. Figure 5 shows an alignment of the nucleotide and amino acid sequences of HVDAC1 and HVDAC2. The HVDAC2 sequence contains a single long open reading frame with two ATGs near the 5' end. The first ATG is at nucleotide 63 and the second at position 96 of the cDNA. The ATG at position 63 shows a good (8/9) match with the start codon consensus sequence [CCA/GCCATG(G)], consistent with the proposition that this is the translation start site, although the second ATG corresponds to the position of the proposed start codon in HVDAC1 and the start codons of *N. crassa* and *S. cerevisiae* genes (Fig. 6). Use of the 5'-most ATG as the start sites would generate an HVDAC2 protein with an additional 11 amino acids at its N-terminus compared to HVDAC1. Comparison of the amino acid sequences encoded by HVDAC1 and HVDAC2 indicate that they are 75% identical; most differences being the result of conservative changes.

Figure 4. Restriction maps of HVDAC1 and HVDAC2 cDNAs

The restriction maps of HVDAC1 and HVDAC2 cDNA clones are shown. The bold lines indicate the coding region of each cDNA.

HVDAC1



HVDAC2

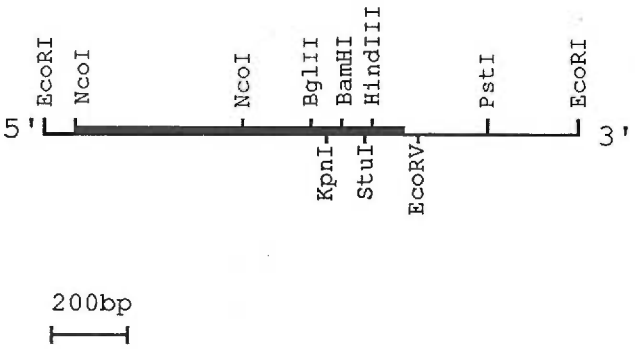


Figure 5. Nucleotide and amino acid sequences of HVDAC1 and HVDAC2

HVDAC2 amino acid and DNA sequences are shown in the upper and second lines. The third and the lower lines show the HVDAC1 DNA sequence and amino acid sequence. Dashes in HVDAC1 amino acid sequences indicate identity with HVDAC2.

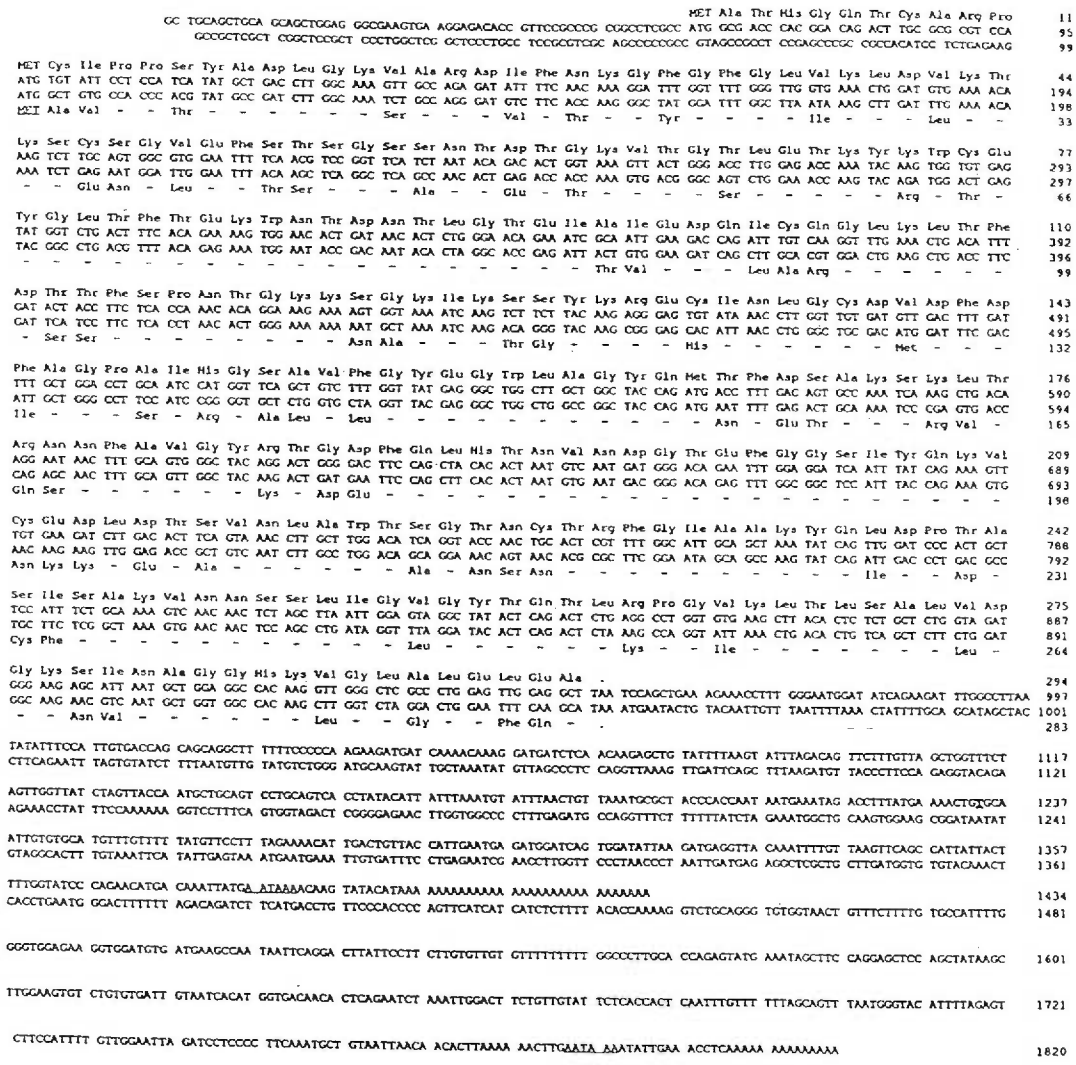
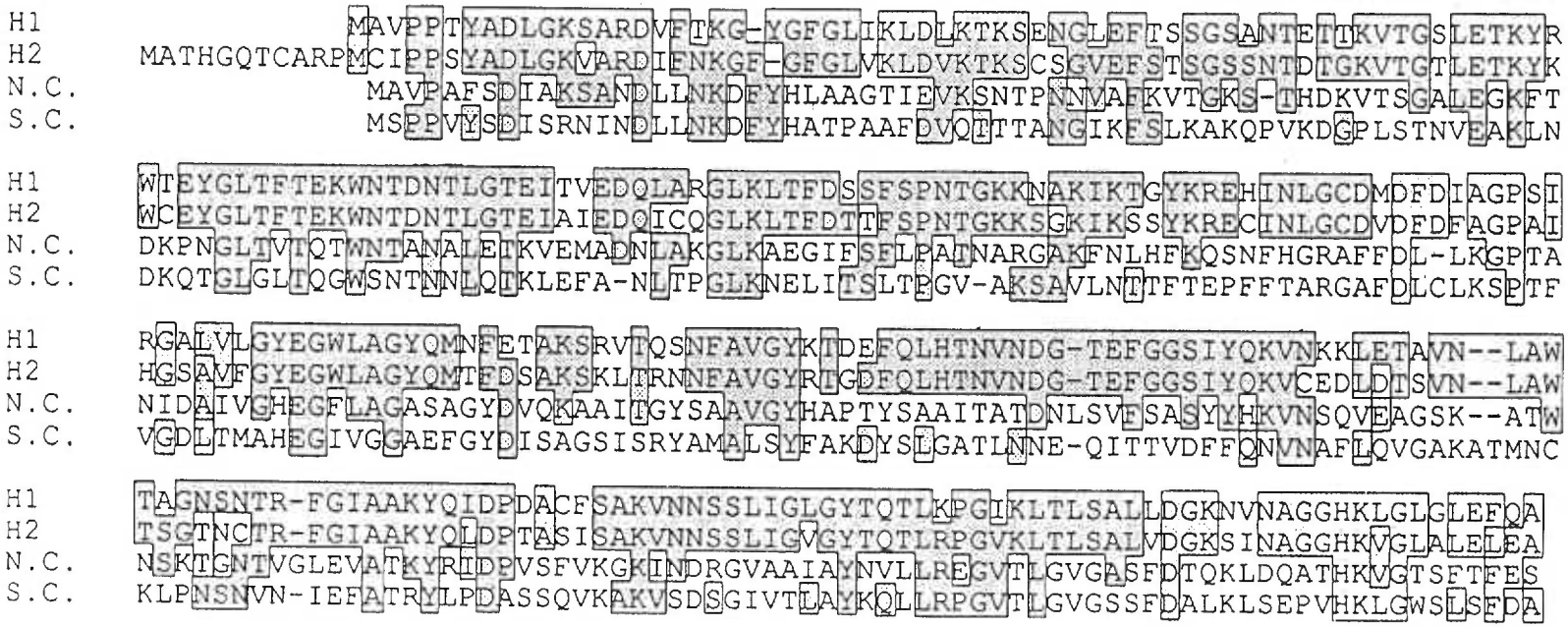


Figure 6. Comparison of the primary amino acid sequence of fungal and HVDAC proteins.

Alignment of the amino acid sequences of HVDAC1 (H1) and HVDAC2 (H2) with those of the yeast *Saccharomyces cerevisiae* (S.C.) and *Neurospora crassa* (N.C.). Identical residues are boxed. Dashes indicate gaps of the amino acid sequence introduced to optimize alignments.


H1
H2
N.C.
S.C.
H1
H2
N.C.
S.C.
H1
H2
N.C.
S.C.
H1
H2
N.C.
S.C.

Comparison of the sequence motifs present in HVDAC1, HVDAC2 and yeast VDAC revealed that the overall pattern of putative transmembrane, sided β strands is preserved in spite of the low level of sequence identity between the fungal and human sequences. This suggests that human VDAC has the similar conformation to that of proposed for the yeast VDAC channel (Blachly-Dyson, Zambronicz et al. 1993).

HVDAC1 and HVDAC2 were subcloned into a yeast expression vector so that they were expressed as fusion proteins with the yeast VDAC molecule; the first 9 amino acids of yeast VDAC fused to codon 11 of HVDAC1 and codon 22 of HVDAC2. Both expression plasmids were introduced into a yeast strain lacking the endogenous VDAC gene (m22-2). This strain is viable but temperature sensitive for growth on the glycerol based medium since colonies are formed only at 30° C but not at 37° C. Expression of wild type yeast VDAC gene corrected the growth defect of m22-2 strain at the non-permissive temperature. Expression of HVDAC1 and HVDAC2 fusion proteins in these cells complimented the temperature-sensitive growth defect, suggesting that each human VDAC can functionally substitute for the yeast VDAC protein. Further, when expressed and purified from yeast lacking endogenous VDAC genes, each human protein can form channels in planar lipid bilayers with physiological properties expected for VDAC (Table 1).

Expression of HVDAC1 and HVDAC2 in a variety of cell lines and human tissues was assessed by Northern blot and PCR amplification of transcripts. The results indicate that HVDAC1 and HVDAC2 are expressed in all cell types and tissues tested. VDAC has also been demonstrated to be the mitochondrial binding site for hexokinase I. To determine the ability of HVDAC1 and HVDAC2 to bind hexokinase, mitochondria prepared from yeast strains expressing HVDAC1 and HVDAC2 were incubated with purified

Table 1. Physiological properties of HVDAC1 and HVDAC2 expressed in yeast

Physiological properties of HVDAC1 and HVDAC2 expressed in yeast were characterized. Value for yeast VDAC were taken from Forte *et al.* (1987), Blachly-Dyson *et al.*, (1990), or Peng *et al.*, (1992). Selectivities calculated from reversal potential values according to the Goldman/Hodgkin/Katz equation.

	HVDAC1	HVDAC2	YEAST
Single-channel conductance (nS) in 1M KCl	4.1 ± 0.1 (6)	4.0 ± 0.2 (6)3	4.2 ± 0.1 (3)
Single-channel conductance (nS)	1.87 ± 0.05 (4)	1.76 ± 0.5 (3)	1.80 ± 0.06 (4)
Reversal Potential in 0.1M KCl vs 1M KCl	11.1 ± 0.6 (5)	10.9 ± 0.2 (2)	11.0 ± 0.2 (6)
Selectivity P(Cl-)/P(K+)	1.8	1.8	1.8

hexokinase and binding assessed. HVDAC2 binds rat brain hexokinase to the same extent as mitochondria prepared from *vdac-* cells, while HVDAC1 binds to hexokinase significantly above these background levels (Blachly-Dyson, Zambronicz et al. 1993). Thus, these two human isoforms appear to differ in their ability to bind hexokinase.

Additional human VDAC isoforms

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, Hajek et al. 1993). The HVDAC2' partial cDNA was isolated during the cloning of the human B lymphocyte-specific gene mb-1 as a chimerical clone. This chimerical clone contained 120 bp of the 3'-untranslated region of mb-1 and 75 bp of HVDAC2' sequence. Full length clones of HVDAC2' were obtained in further screens which identified clones that hybridized with the chimerical clone and not an oligonucelotide representing the human mb-1 gene. This cDNA clone contains an open reading frame with two ATG sites near its 5' end. *In vitro* translation of the HVDAC2' cDNA generated two proteins of 32 kDa and 36 kDa. This result suggests the alternate use of start codons during *in vitro* translation. The full length HVDAC2' protein (36 kDa; use of 5' most ATG) differs from the HVDAC2 by the replacement of the 11 amino acid N-terminal extension present in HVDAC2 with a distinct 26 amino acid extension relative to HVDAC1 (Fig. 7). The short form (32 kDa; use of second ATG) is predicted to have the same molecular weight as HVDAC1. This report also suggested differences at the C terminus of HVDAC2' when compared to HVDAC2. However, these differences are due to sequencing errors in the nucleotide sequence reported for HVDAC2'. Thus, the proteins encoded by the HVDAC2' cDNA are predicted to have the same C terminal amino acid

Figure 7. Comparison of amino terminal sequences of HVDAC isoforms

Comparison of the amino terminal sequence of proteins encoded by HVDAC1, HVDAC2 and HVDAC2' cDNAs. Residues 1-8 of HVDAC1 are aligned with residues 1-19 of HVDAC2 (Blachly-Dyson, Zambronicz et al. 1993) and residues 1-34 of HVDAC2' (Ha, Hajek et al. 1993).

HVDAC1 MAVPPTYA$_{8}$

HVDAC2 MATHGQTCARPMCIPPSYA$_{19}$

HVDAC2' MSWCNELRLPALKQHSIGRGLESHITMCIPPSYA$_{34}$

sequence as that reported for HVDAC2. The HVDAC2' cDNA was detected from all tissues and cell lines examined (Ha, Hajek et al. 1993).

During gene mapping of HVDAC1 and HVDAC2, two additional HVDAC1-like genes were identified by PCR (Blachly-Dyson, Baldini et al. 1994). One gene mapped to chromosome 12 (HVDAC3) and was found to have 95% nucleotide sequence identity to HVDAC1 between PCR primers used for amplification. In addition, this gene appears to have a stop codon within the coding region (Fig. 8) relative to HVDAC1, suggesting that it could be a pseudo gene. A fourth gene (HVDAC4) mapped to chromosome 1 and was also found to be 95% identical to HVDAC1 at the nucleotide level. Similar techniques were used to map the location of the genes encoding HVDAC1 (X-chromosome) and HVDAC2/HVDAC2' (chromosome 5).

The results outlined above indicated that multiple VDAC genes are present in mammals which can potentially encode up to 5 isoforms of the VDAC protein. Two human genes, HVDAC1 and HVDAC2, appear to encode at least 3 distinct proteins which differ most significantly over their initial amino terminal regions. These results have led to the hypothesis that the distinct amino termini present in HVDAC1, HVDAC2 and HVDAC2' lead to the targeting of these molecules to different cellular compartments.

Subcellular localization of human VDAC isoforms

Human VDAC isoforms share extensive amino acid homology (around 75% between HVDAC1 and HVDAC2) and do not differ sufficiently in any single domain to allow the generation of antibodies which can distinguish each isoforms. In order to analyze the subcellular localization of each isoform, we took advantage of the availability of both monoclonal and polyclonal antibodies which specifically recognize epitopes of defined amino

Figure 8. Alignment of the nucleotide sequences of HVDAC1, 2, 3 and 4

Sequence comparison of HVDAC sequences. Sequences of cloned PCR fragments from HVDAC3 (line2) and HVDAC4 (line3) are aligned with the homologous portion of the HVDAC1 gene (top) and HVDAC2 (bottom). Nucleotides identical HVDAC1 are indicated by dots, except in termination codons, where they are italicized. Termination codons are underlined (Taken from Blachly-Dyson, Baldini et al., 1994).


HVDAC1 GCCTGCTTCTCGGCTAAAGTGAACAACTCCAGCCTGATAGGTTTAGGA
HVDAC3 ..T.....T.....
HVDAC4 AG.....T.....G.....
HVDAC2 ..T.C.A.T..T..A.....C.....T...T.A..T..AG....C
231 235 240 245
HVDAC1 TACACTCAGACTCTAAAGCCAGGTATTAAACTGACACTGTCAGCTCTT
HVDAC3 TAA.....C.....C.....
HVDAC4 .....C.....A.....C...T.....
HVDAC2 ..T.....G.G...T...G.G..G..T.....C..T.....G
250 255 260
HVDAC1 CTGGATGGCAAGAACGTCAATGCTGGTGGCCACAAGCTTGGTCTAGGA
HVDAC3 ......CT.....G....G.....C..T.....
HVDAC4 .....G.....T....C
HVDAC2 G.A.....G....G.A.T.....A.....G....G..C.CC
265 270 275
HVDAC1 CTGGAATTTCAAGCATAAATGAATACTGTACAATTGTTTAATTTTAAA
HVDAC3 .....TAA.....C.....
HVDAC4 .....TAA.....
HVDAC2 .....G..GG.G..TTAATCC.GCTGAAAGA..CCT..GGGAA.GG.T
280
HVDAC1 CTATTTTGCAGCATAGCTACC-TTCAGAATTTAGTGTATCTTTTAATG
HVDAC3 .....C.....G....G..C.....
HVDAC4 .....-.....C.....G.....
HVDAC2 A.CAGAA.ATTTGGCCT..ATA.ATTTCCA..GTGACCAGCAGC.GGC
HVDAC1 TTGTATGTCTGGGATGCAAGTATTGCTAAATAT
HVDAC3 .....
HVDAC4 .....C.....A.....
HVDAC2 ..T.T.CC.CCAAGAAG.T.ATCAAAAC..AGG

acid sequence. In this study two such epitopes were used, the synthetic FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) and an epitope derived from the human influenza hemagglutinin protein (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala, HA). Constructs were made as described in "Material and Methods" and outlined in Figure 2. Novel ClaI sites were generated at the stop codons of HVDAC1, HVDAC2 and HVDAC2' by PCR. Double stranded DNA cassettes encoding the FLAG (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) and HA (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala) epitopes were constructed containing 5' ClaI sites and 3' XhoI sites followed by a stop codon. These cassettes were inserted between the newly created ClaI sites at the C terminus of each gene and a vector XhoI site to create cDNAs encoding HVDAC1, HVDAC2 and HVDAC2' epitope-containing molecules (Fig. 2).

Expression of epitope-tagged VDAC in yeast

Our previous characterization of HVDAC1 and HVDAC2 proteins has demonstrated that each is capable of complementing the growth defects associated with deletion of the endogenous yeast VDAC gene (Blachly-Dyson, Zambronicz et al. 1993). Deletion of yeast VDAC gene results in cells that are viable on glucose-based media at either 30° C or 37° C but fail to grow at 37° C on non-fermentable carbon sources such as glycerol. Although the physiological basis of this phenotype is currently unknown, these growth defects can be corrected by expression of a plasmid-based yeast VDAC gene or by expression of either HVDAC1 or HVDAC2 cDNAs (Blachly-Dyson, Zambronicz et al. 1993). These results demonstrate that each human protein can functionally replace the yeast VDAC gene in yeast cells. To determine whether introduction of either epitope tag at the C terminus of the human proteins significantly altered their function, cDNAs encoding FLAG or HA

tagged HVDAC1, HVDAC2 or HVDAC2' proteins were transformed into yeast cells lacking an endogenous yeast VDAC gene. As shown in Figure 9, introduction of either epitope into HVDAC1, HVDAC2 or HVDAC2' has no effect on the ability of these human proteins to complement the growth defects present in this strain. All the transformants grow at both 30° C and 37° C on glycerol based medium. In addition, epitope-tagged HVDAC molecules purified from yeast are able to form channels when incorporated into planar phospholipid bilayers (not shown).

To examine expression in yeast biochemically, mitochondrial membranes from strains containing each tagged-HVDAC cDNA were prepared and separated on SDS-polyacrylamide gels. Separated proteins were then transferred to nitrocellulose and bound proteins probed with antibodies to the appropriate epitope. As shown in Figure 10 for FLAG-HVDAC molecules, tagged proteins are efficiently targeted to yeast mitochondria and each has the appropriate relative mobility given the expected differences in N termini; HVDAC2 migrates more slowly than HVDAC1, consistent with the additional N terminal 11 amino acids present in HVDAC2 and HVDAC2' migrates more slowly than HVDAC2, consistent with the additional N terminal residues present in HVDAC2' relative to HVDAC2.

Expression of epitope-tagged VDAC in mammalian cells

Stable transfection

cDNA encoding FLAG-tagged HVDAC1 and HVDAC2 were subcloned into a mammalian expression vector pRC/RSV at the multicloning site. The pRC/RSV vector contains a Rous Sarcoma Virus LTR promoter, a bovine growth hormone polyadenylation signal, a multicloning site, and a neomycin resistance gene expressed from the SV40 early promoter allowing for the

Figure 9. Differential growth of strains lacking VDAC and strains expressing epitope-tagged HVDAC molecules on media containing glycerol

A yeast strain in which the chromsomal VDAC gene was deleted (Δ) (Blachly-Dyson, Peng et al. 1990) was transformed with plasmids mediating the expression of the wild-type yeast VDAC (YVDAC) and individual FLAG-tagged (A) or HA-tagged (B) HVDAC proteins. The resulting strains were then streaked on media containing 2% glycerol as the sole carbon source and incubated at the indicated temperatures.

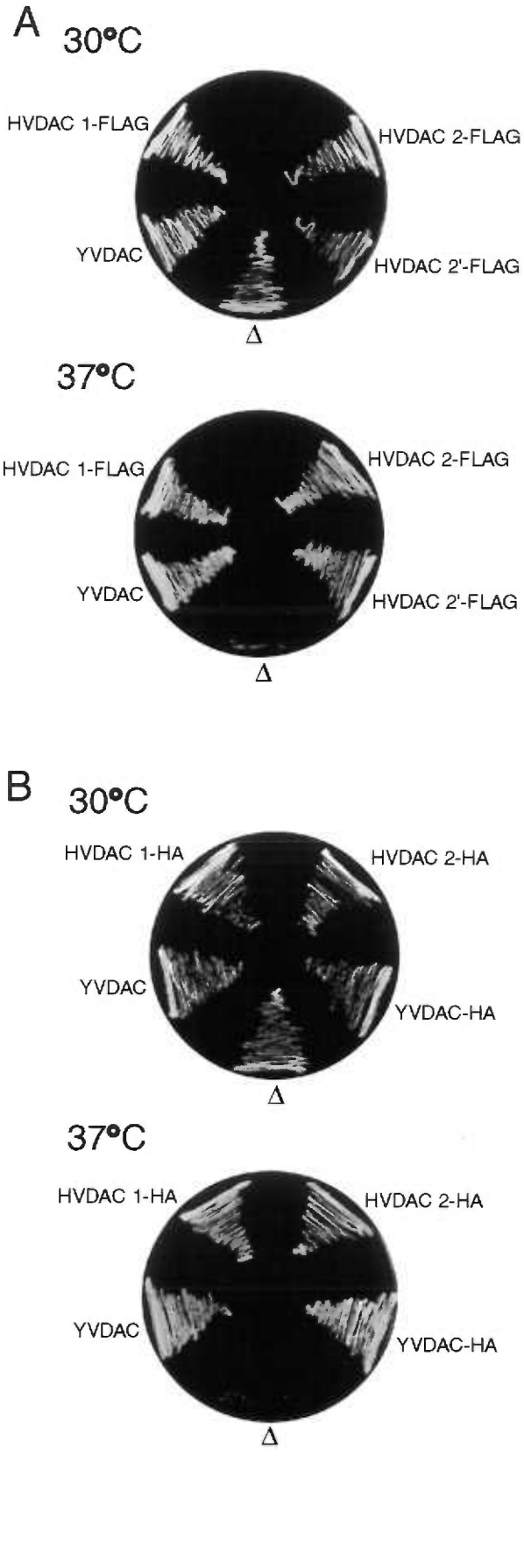
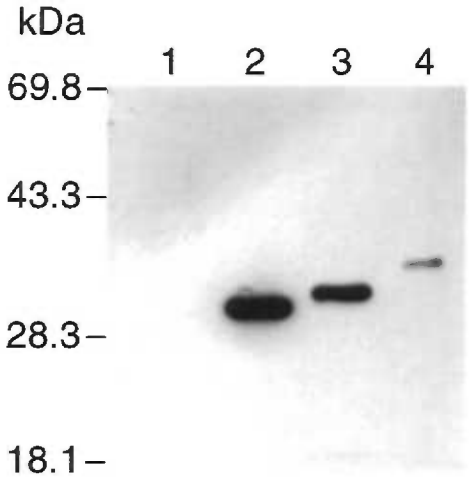

A
30°C
HVDAC 1-FLAG
HVDAC 2-FLAG
YVDAC
HVDAC 2'-FLAG
Δ
37°C
HVDAC 1-FLAG
HVDAC 2-FLAG
YVDAC
HVDAC 2'-FLAG
Δ
B
30°C
HVDAC 1-HA
HVDAC 2-HA
YVDAC
YVDAC-HA
Δ
37°C
HVDAC 1-HA
HVDAC 2-HA
YVDAC
YVDAC-HA
Δ

Figure 10. Immunoblot of yeast mitochondrial membranes prepared from *vdac*⁻ strain and strains expressing HVDAC-FLAG proteins.

Immunoblot of yeast mitochondrial membranes prepared from cells lacking VDAC (lane1), or expressing HVDAC1 (lane 2), HVDAC2 (lane 3) and HVDAC2' (lane 4) each containing the FLAG epitope. The blot was probed with mouse m2 anti-FLAG monoclonal antibodies (3 μg/ml). Mitochondrial membranes and immunoblots were prepared as described in the Methods. Each lane contains 10 μg protein as determined in Material and Methods.

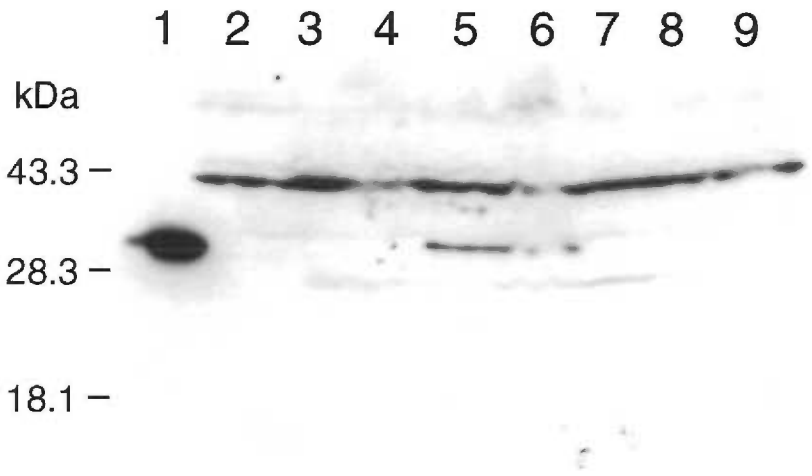

kDa
1
2
3
4
69.8
43.3
28.3
18.1

selection of stable transformants. Ltk$^-$ cells were transfected with these constructs and 48-72 hours after transfection, cells plated into 150 mm plates and grown in DMEM media containing 700 μg/ml G418. Individual colonies formed under G418 selection were picked and expanded in DMEM medium containing G418. Total protein was extracted from neo^r cell lines, loaded on an SDS-polyacrylamide gels and proteins transferred to nitrocellulose. A monoclonal antibody against FLAG epitope, m2, was used to detect the expression of FLAG-tagged HVDACs on Western-blots. Two out of twelve HVDAC1-FLAG transfectants shown faint positive expression of HVDAC1-FLAG (Fig. 11). No HVDAC2-FLAG expression was detected by Western-blot (data not shown).

To determine whether HVDAC2-FLAG cDNAs have been incorporated into the genomic DNA of stable transfectants, DNA samples were isolated from HVDAC2-FLAG transfected lines and the presence of the FLAG sequence detected by PCR using sense oligonucleotides corresponding to residue 721 to 743 (R873) of HVDAC2 cDNA and an antisense oligonucleotides corresponding to the FLAG sequence (S529) (Fig. 12). In four out of 10 neo^r HVDAC2-FLAG transfectants, bands of the appropriate size (230 bp) were amplified (Fig. 13). As a positive control, oligo R873 and an oligo corresponding to an antisense sequence representing residues 1093-1114 of HVDAC2 (R874) were used to amplify the HVDAC2 homolog in Ltk$^-$ cells. Use of these primers resulted in the amplification of a 393 bp fragment from untransfected Ltk$^-$ cells. These results suggest that HVDAC2-FLAG sequences were incorporated into the genomic DNA of host cells yet these genes were either not expressed or expressed at very low levels. To characterize stable transfectants further, two HVDAC1-FLAG transfected, neo^r clones were

Figure 11. Immunoblots of total protein prepared from neo^r, HVDAC1-FLAG transfected Ltk$^-$ cells

Samples were prepared from untransfected Ltk$^-$ cells (lane 2) and HVDAC1-FLAG transfected neo^r cell lines (lane 3-14) as described in "Material and Methods". Each lane contains proteins extracted from $2x10^6$ cells. 10 μg of protein prepared from yeast mitochondria expressing HVDAC1-FLAG (lane 1) was also loaded on gel as a positive control. Blot was probed with mouse m2 anti-FLAG (1.7 μg/ml) monoclonal antibodies.


1 2 3 4 5 6 7 8 9
kDa
43.3 –
28.3 –
18.1 –

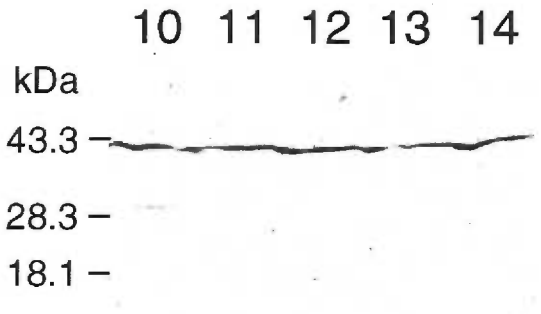

10 11 12 13 14
kDa
43.3 –
28.3 –
18.1 –

Figure 12. Schematic of the PCR reactions used to detect HVDAC2-FLAG in the genome of neo^r, transfected Ltk$^-$ cells

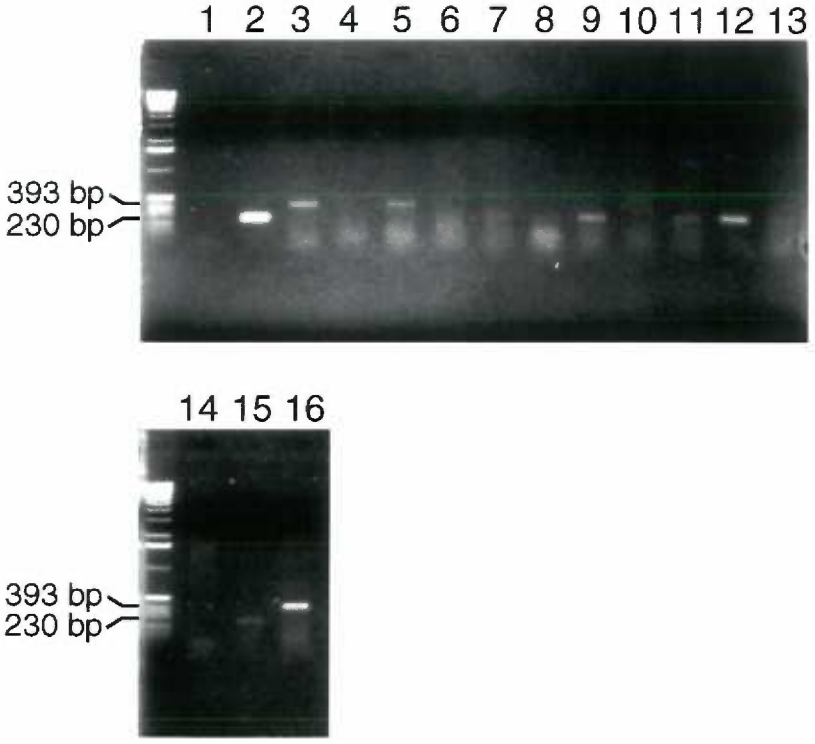
Line: vector; open box: coding region of the HVDAC2 cDNA and putative mouse homolog of HVDAC2; filled box: 5' and 3' untranslated regions; wavy line: FLAG epitope; arrows: primers.

HVDAC2-FLAG

mouse homolog of HVDAC2

Figure 13. PCR analysis of HVDAC2-FLAG integration

PCR amplification of HVDAC2-FLAG sequences from genomic DNA isolated from neo^r, HVDAC2-FLAG transfected Ltk cells. The PCR products were analyzed on a 1% agarose gel. The primers used in PCR reactons are either R873 and S529 (lane 1,2,4 and 6-15) or R874 and R874 (lane 3, 5 and 16). Lane 1 contains the products of a PCR without DNA template; lane 2 contains the products of a PCR using the HVDAC2-FLAG cDNA construct as the template. Product of PCR using templates isolated from untransfected Ltk^- cells (lane 3 and 4); clone $RL3_2$ (lane 5 and 6); clone $RL3_3$ (lane 7); clone $RL3_4$ (lane 8); clone $RL3_5$ (lane 9); clone $RL3_6$ (lane 10 and 16); clone $RL3_7$ (lane 11); clone $RL3_8$ (lane 12); clone $RL3_9$ (lane 13); clone $RL3_{10}$ (lane 14) and clone $RL3_{12}$ (lane 15). The position of 230bp and 393 bp amplification products are indicated.


1 2 3 4 5 6 7 8 9 10 11 12 13
393 bp
230 bp
14 15 16
393 bp
230 bp

examined by light level immunofluorescence with m2 anti-FLAG antibody as described in the Material and Methods. Less than 1% of cells in a culture showed a positive perinuclear punctate staining, although the pattern of staining in positive cells is similar to that observed in positive, transiently transfected cells (see below).

The results outlined above indicate that epitope-tagged HVDAC proteins were not expressed in mammalian cells stably transfected with these constructs. Although the reasons for this are unknown, they do demonstate that alternate expression systems need to be employed in order to investigate the subcellular location of each epitope-tagged HVDAC isoform.

Transient transfection

Since stable transfection is not feasible, a transient expression system was used in the studies that follow. Epitope-tagged HVDAC cDNA were subcloned into the multicloning site of a mammalian expression vector pCD-PS containing a SV40 promoter and SV40 late polyadenylation signal. In all the experiments, cells were either fixed or harvested within 48 hours after transfection. To determine the extent of transfection, populations of HVDAC-FLAG transfected cells were analyzed by immunocytochemical techniques using the m2 anti-FLAG antibody. By counting positively stained cells, the percentage of cells successfully transfected with epitope-tagged HVDAC cDNA constructs was obtained. Roughly 20-40% of transfected cells expressed either epitope-tagged form of HVDAC1 and HVDAC2 while roughly 10% of the transfected cells expressed epitope-tagged forms of HVDAC2'.

Western-blot analysis of FLAG-tagged HVDAC expression in transfected COS7 cells.

Total protein samples were prepared from nontransfected COS7 cells, COS7 cells transfected with pCD/PS vector alone, and COS7 cells transiently transfected with FLAG-tagged HVDAC1, HVDAC2 and HVDAC2' cDNAs as described in the Materials and Methods. Protein samples were resolved by SDS-polyacrylamide gels and transferred to nitrocellulose. Bound proteins were then probed with the m2 anti-FLAG antibody. Single proteins of appropriate size were present in cells expressing FLAG-tagged forms of HVDAC1 and HVDAC2. Two bands were detected in cells transfected with epitope-tagged forms of HVDAC2' (Fig. 14). HVDAC2' bands of similar relative molecular weight appear to arise by use of alternate 5' translational initiation points following *in vitro* translation of HVDAC2' transcripts. These transfectants are viable for at least 48 hours following transfection. These results demonstrate that epitope-tagged forms of each HVDAC isoform can be expressed in COS7 cells following transient transfection.

Localizing HVDAC isoforms by subcellular fractionation

The subcellular location of each epitope-tagged HVDAC molecule was initially determined by differential centrifugation of extracts prepared from COS7 cells transfected with HVDAC constructs expressing FLAG-tagged molecules. Aliquots of fractions enriched for soluble components of the cytoplasm (high speed supernatant), mitochondria (low speed pellet) and other membrane components including plasma membrane (high speed pellet) were then separated on SDS-polyacrylamide gels. Equal amounts of protein from each fraction were loaded in each lane. Resulting Western-blots were subsequently probed with m2 anti-FLAG antibodies. As shown in Figure 15, HVDAC-FLAG proteins were detected exclusively in low speed pellets expected to be enriched in mitochondria and not in soluble cytoplasmic

Figure 14. Immunoblots of total protein prepared from transfected COS7 cells Samples prepared from untransfected cells (lane 1), cells transfected with vector alone (lane 2), and vector containing HVDAC1-FLAG (lane 3) HVDAC2-FLAG (lane 4) and HVDAC2'-FLAG (lane 5) constructs. Also shown for comparison are samples prepared from yeast mitochondria containing HVADC1-FLAG (lane 6), HVDAC2-FLAG (lane 7) and HVDAC2'-FLAG (lane 8). Blot was probed with mouse anti-FLAG (1.7 μg/ml) monoclonal antibodies (m2). Each lane contains protein from an equal number of transfected cells.

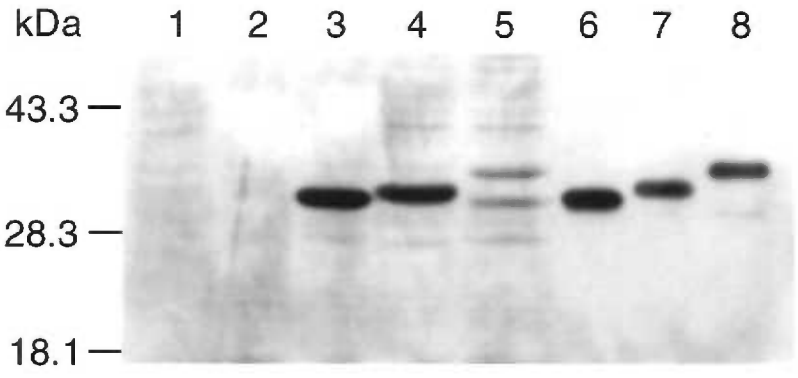

kDa
1
2
3
4
5
6
7
8
43.3
28.3
18.1

Figure 15. Immunoblots of subcellular fractions prepared from transfected COS7 cells

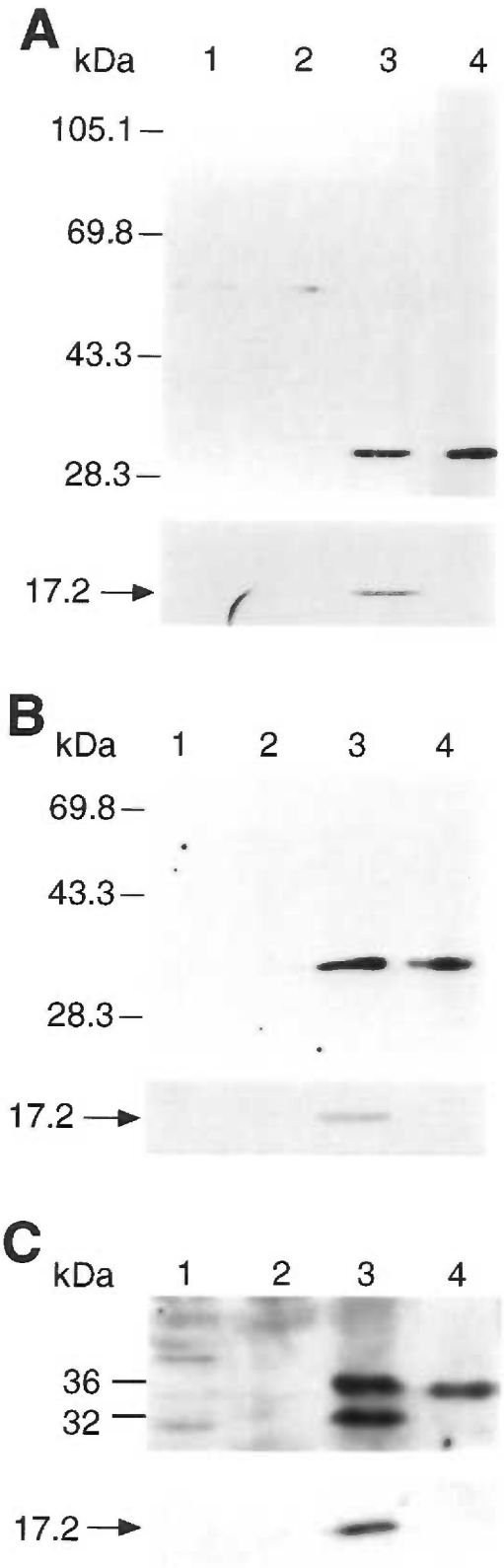
Fractions were prepared as described in Material and Methods. In each case, lanes represent samples of high speed supernatant (lane 1), high speed pellet (lane 2), low speed pellet (lane 3) and samples prepared from yeast mitochondria containing the appropriate FLAG-tagged HVDAC protein (lane 4). The amount of protein loaded in each case was varied due to differences in transfection effeciency in specific populations of cells.

A. Fractions prepared from cells transfected with HVDAC1-FLAG constructs. Each lane contains 30 μg protein.

B. Fractions prepared from cells transfected with HVDAC2-FLAG constructs. Each lane contains 50 μg protein.

C. Fractions prepared from cells transfected with HVDAC2'-FLAG constructs. Each lane contains 100 μg protein.

Blots were probed with mouse anti-FLAG monoclonal antibodies (3 μg/ml) or monoclonal antibodies to subunit IV of mitochondrial cytochrome C oxidase. Arrow indicates the position of subunit IV (17.2 kDa).

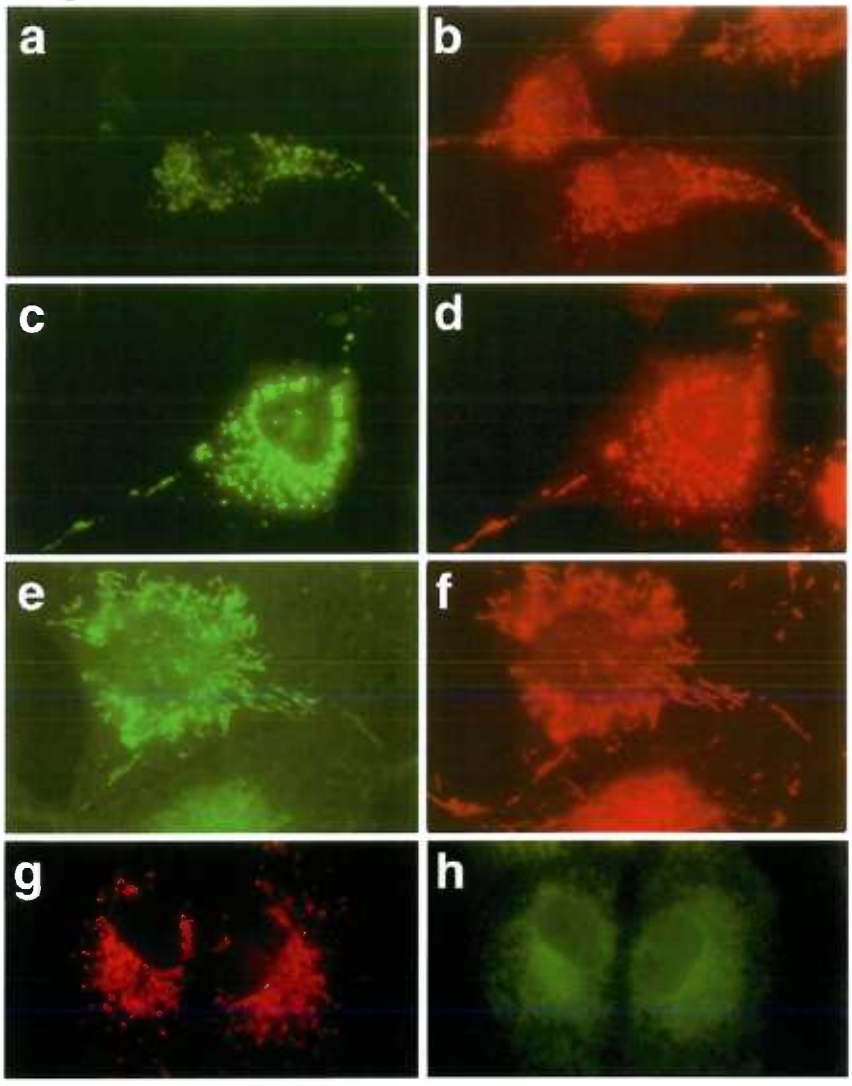

A
kDa
1
2
3
4
105.1
69.8
43.3
28.3
17.2
B
kDa
1
2
3
4
69.8
43.3
28.3
17.2
C
kDa
1
2
3
4
36
32
17.2

fractions or in high speed pellets containing plasma membrane and other cellular membranes. To identify mitochondrial fractions, blots were subsequently re-probed with a monoclonal antibody against the subunit IV of cytochrome C oxidase. This mitochondrial marker was also detected exclusively in low speed pellets. This analysis suggests that epitope-tagged HVDAC1, HVDAC2 and HVDAC2' molecules are located in cellular fractions enriched in mitochondria and not in other cellular compartments.

Light microscopic localization of HVDAC isoforms in COS7 cells

Mitochondrial localization of epitope-tagged HVDAC molecules was confirmed by indirect immunofluorescence microscopy. COS7 cells were seeded on glass coverslips and transfected with cDNAs encoding epitope-tagged forms of HVDAC1, HVDAC2 and HVDAC2'. After fixation, cells were incubated with appropriate monoclonal antibodies and a rabbit polyclonal antibody raised against the mitochondrial cytochrome C oxidase complex to identify the location of mitochondria. As shown in Figure 16 for cells expressing FLAG-tagged HVDAC proteins, antibodies to the FLAG epitope specifically label the same subcellular structures as antibodies to mitochondrial cytochrome C oxidase. In both cases, staining appears exclusively in punctate or vermiform organelles distributed throughout the cytoplasm with higher concentrations in perinuclear areas, as expected for mitochondria. The appearance of the stained organelles appears to alternate between punctate and vermiform in a cell specific fashion independent of the antibody used for staining (FLAG or cytochrome C oxidase). In addition, close examination of stained organelles at this level suggests that HVDAC isoforms are unevenly distributed within an individual mitochondrion compared to cytochrome C oxidase staining (for example, compare Fig. 16*e* and Fig. 16*f*).

Fig 16

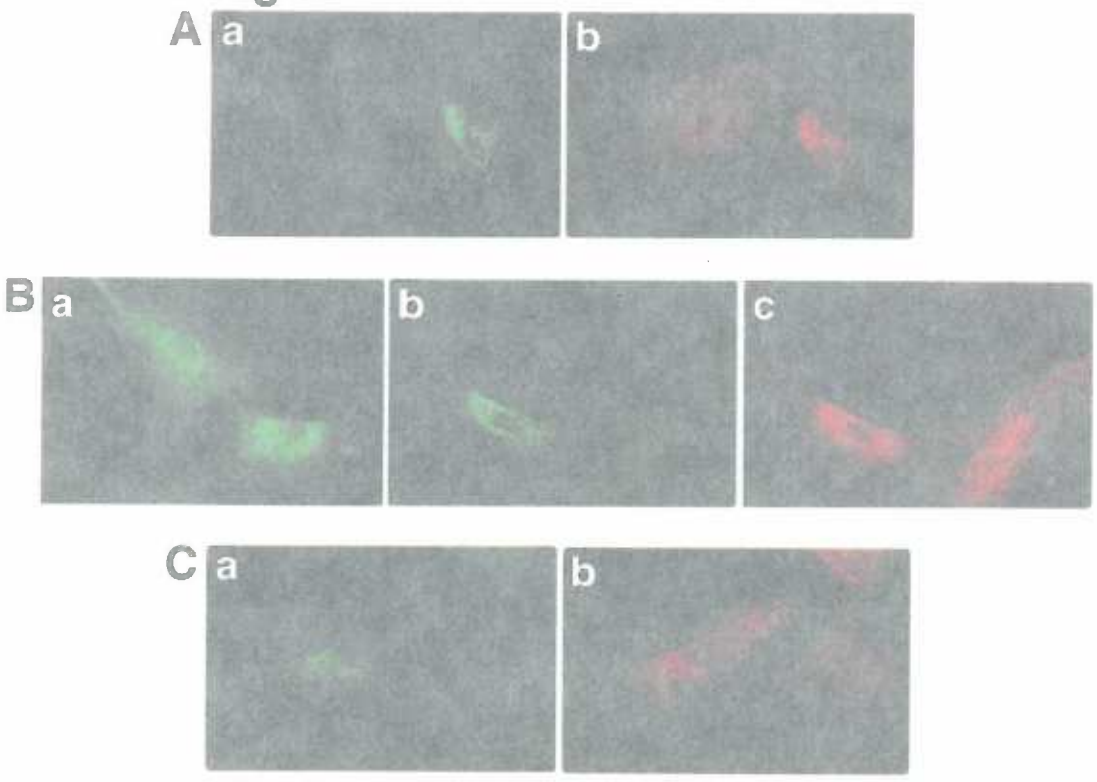


To compare the distribution of HVDAC-FLAG in cells with that of other cell organelles such as rough ER, antibodies to the signal sequence receptor (SSR) were used to identify the position of the rough endoplasmic reticulum (ER). The SSR staining is clearly different from the anti-FLAG staining and is present in a diffuse, reticulate pattern characteristic of the ER (Fig. 16 *h*). In no case is there significant plasma membrane-associated staining in cells transfected with any FLAG-tagged HVDAC constructs nor is plasma membrane staining more intense in transfected cells when compared to untransfected cells (Fig. 16). Similar results were obtained with COS7 cells transfected with HA-tagged HVDAC constructs (not shown).

Light microscopic localization of HVDAC isoforms in rat astrocytes

A recent report of plasma membrane-localized VDAC molecules suggested the presence of homologs of HVDAC1 in the plasma membrane of rat astrocytes (Dermietzel, Hwang et al. 1994). To examine the subcellular location of epitope-tagged HVDAC molecules in these cells and to investigate the possibility that cell type specific differences may exist in the targeting of these molecules, rat astrocytes were transiently transfected with constructs encoding FLAG-tagged HVDAC1, HVDAC2 and HVDAC2' molecules. The cells were then processed for indirect immunofluorescence as described above for COS7 cells (paraformaldehyde fixation) or as described by Dermietzel et al. (Dermietzel, Hwang et al. 1994) (ethanol fixation) (Material and Methods). Following the fixation, the cells were incubated with mouse monoclonal antibodies to the FLAG epitope and the rabbit polyclonal antibody raised against the cytochrome C oxidase complex to identify the location of mitochondria. As shown in Figure 17A for astrocytes expressing FLAG-tagged

Fig 17



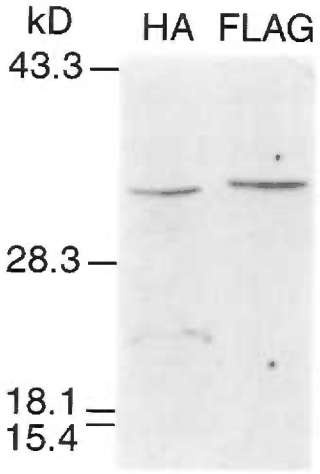
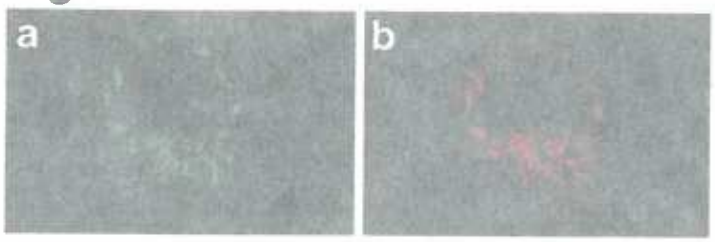
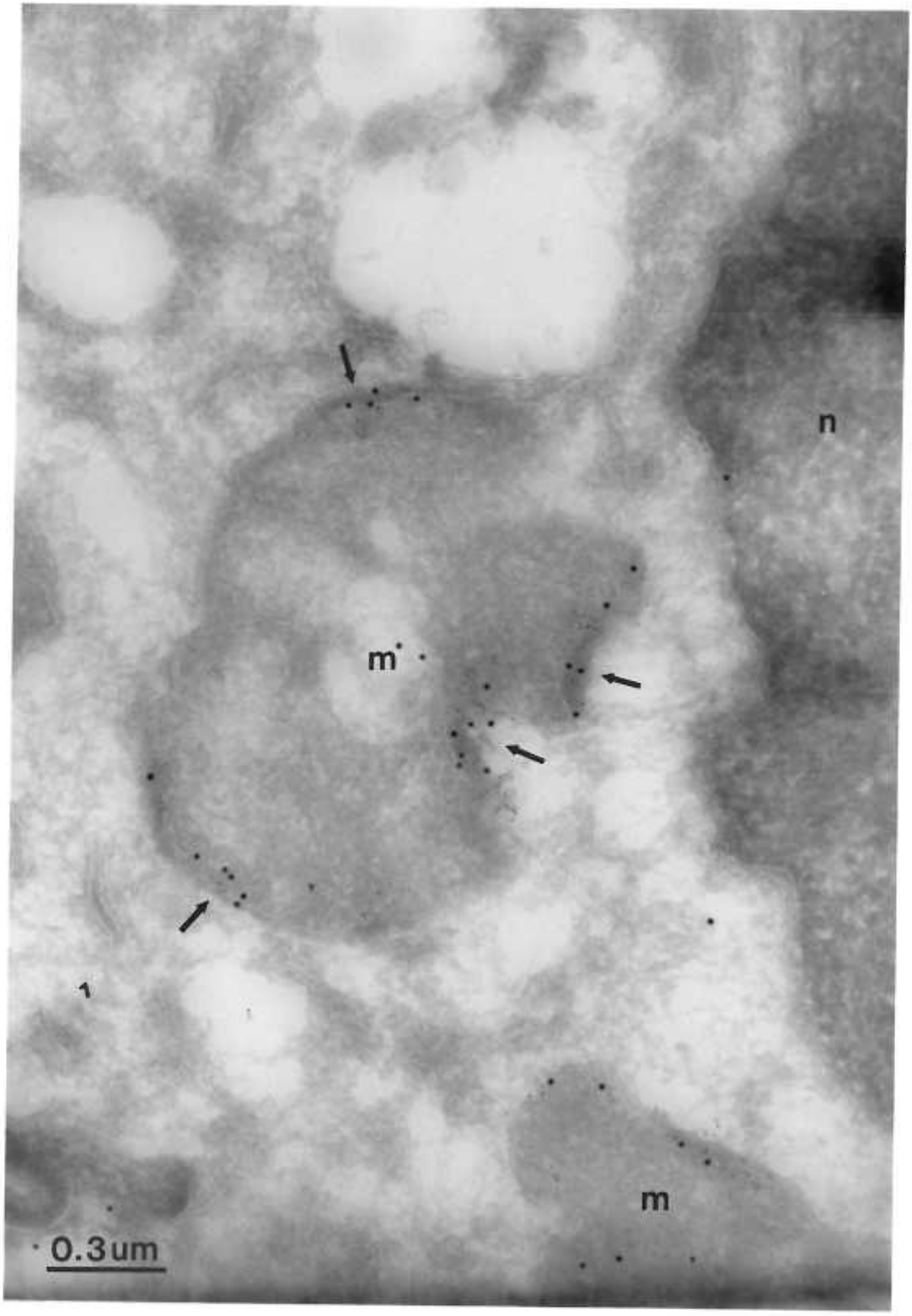

kD
HA
FLAG
43.3
28.3
18.1
15.4

Figure 20. Colocalization of epitope-tagged HVDAC1 and HVDAC2 in co-transfected COS7 cells

COS7 cells were co-transfected with HVDAC1-HA and HVDAC2-FLAG. Transfected cells were stained with mouse anti-HA monoclonal antibodies (*a*) and a rabbit anti-FLAG polyclonal antibody (*b*).

Fig 20




n
m
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0.3um

Conclusion and Discussion

VDAC is thought to play a crucial role in the transport of metabolites between mitochondria and the cytoplasm since many metabolites can pass through the channel. When the channel is in the open state, the conduction pathway formed is slightly anion-selective. Most substrates and products of oxidative phosphorylation such as pyruvate, ADP and ATP are negatively charged. Thus, the existence of an anion-favoring, large channel in the outer mitochondrial membrane fits nicely with a role for VDAC as the regulator of the traffic of these molecules into and out of the mitochondria. Although the voltage-dependent channel activities of VDAC have been studied extensively *in vitro*, direct demonstration of the physiological function of VDAC has been more difficult. The VDAC cDNA clones described in the work presented here provided us a useful tools for investigating the biological function of this family of proteins.

The first human VDAC cDNA clone, HVDAC1, was identified using amino acid information obtained from the protein sequence of VDAC purified from a human B lymphocyte cell line (Blachly-Dyson, Zambronicz et al. 1993). The amino acid sequence of HVDAC1 is identical to that of the purified protein, "Porin 31HL". (Thinnes, Götz et al. 1989). In this study, another human VDAC isoform, HVDAC2, was identified by screening a human liver cDNA library at reduced stringency. This cDNA encodes a protein that is 75% identical to HVDAC1 at amino acid sequence level and has additional 11 amino acids at its N-terminus compared to HVDAC1.

One characteristic of VDAC is the evolutionary conservation of basic channel properties. Both HVDAC1 and HVDAC2 form functional anion-selective channels when incorporated into planar lipid bilayers. In addition,

introduction of plasmid-based HVDAC1 and HVDAC2 cDNAs into *vdac*$^-$ yeast cells complements the temperature-sensitive growth defect resulting from elimination of the endogenous yeast VDAC gene, suggesting that both human VDAC genes can functionally replace the yeast gene in yeast cells. Both HVDAC1 and HVDAC2 have been detected in all cell types and tissues by Northern blot and RT-PCR. The only known functional difference between HVDAC1 and HVDAC2 appears to be that yeast mitochondria containing HVDAC1 bind hexokinase whereas those containing HVDAC2 do not (Blachly-Dyson, Zambronicz et al. 1993).

Although other mammals and higher organisms (e.g., plants) also express a family of VDAC genes, HVDAC1 and HVDAC2 have been characterized in the great detail. For example, these two genes have been mapped to different chromosomes; HVDAC2 to chromsome 5 and HVDAC1 to the X chromosome. In the course of mapping these genes, two additional HVDAC1-like genes were identified, HVDAC3 and HVDAC4. Each is 95% identical to HVDAC1 at nucleotide level and each has been mapped to different chromosome, chromosomes 12 and 1 respectively (Blachly-Dyson, Baldini et al. 1994). Another HVDAC2-like cDNA, HVDAC2', was identified in a human lymphocyte library (Ha, Hajek et al. 1993). This cDNA encodes a protein that is identical to HVDAC2 except for a 26 residue, N-terminal extension compared to HVDAC1 which replaces an 11 residue N terminal extension present in HVDAC2.

Since amino terminal sequences have been demonstrated to target many proteins to appropriate subcellular compartments, differences in the N termini of HVDAC1, HVDAC2 and HVDAC2' raise the possibility that these distinct N termini may lead to targeting of each protein to different cellular locations. Consistent with this hypothesis, a large number of recent reports

hypothesis can easily be tested by staining transfected cells with anti-epitope antibody and following the number of positively stained cells over time. If expression is inhibited, it would be interesting to determine whether inhibition is at the level of transcription or translation. One way to distinguish these two possibilities is to assess the presence of HVDAC-FLAG transcripts by RT-PCR at various time points after transfection.

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 (Fig. 15). 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 (Fig. 16). 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 (Fig. 18). 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. 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 potential 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. These cells have recently been reported to contain rat homologs of HVDAC1 in their plasma membranes by both immunocytochemistry and electrophysiology studies (Dermietzel, Hwang 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 (Fig. 17). In the case of astrocytes, some plasma membrane staining with anti-FLAG antibodies is evident in transfected cells. However, this staining is clearly not specific, since untransfected cells show identical levels of plasma membrane staining. In addition, differences between the results presented here for astrocytes and COS7 cells and those reported by others cannot be attributed to differences in fixation technique since identical mitochondrial staining patterns were observed regardless of cell fixation with paraformaldehyde or ethanol, as used in the study of Dermietzel et al. (Dermietzel, Hwang et al. 1994).

Dermietzel et al. (Dermietzel, Hwang et al. 1994) found VDAC-like channels of over 400 pS in about 2/3 of the patchs prepared from cultured rat astrocytes. In an attempt of record VDAC-like channel activity in these cells, channel in the plasma membrane of cultured rat astrocytes were examined by on-cell patch clamping techniques under conditions that should have revealed VDAC-like channels. No VDAC-like channels were detected in any

of the three patches studied. Although VDAC was never observed, a low-conductance, non-selective channel was detected in two of the three patches, showing that we could easily have detected the large conductance VDAC channels if they had been present. Since only three patches were studied, there is a 3.7% chance that a VDAC-like channel could have been missed if these channels are present in the frequency reported by Dermitzel et al. (2/3 of patches analyzed). Another report of VDAC-like channels in the plasma membrane of rat astrocytes indicates that these channels occur at a lower frequency than reported by Dermietzel et al. [roughly 1/3 of patches examined in astrocytes from 9-to 11-week-old embryos (Jalonen, Johansson et al. 1989)]. Thus, in order to conclusively demonstrate that no VDAC-like channels can be observed in our hands by patch clamping the plasma membrane of mammalian astrocytes, many more patches need to be analyzed.

Cloning of cDNAs for HVDAC2 and HVDAC2' indicate that each is expressed with a different N terminal extension relative to HVDAC1 (Blachly-Dyson, Zambronicz et al. 1993; Ha, Hajek et al. 1993). Consistent with this expectation, the relative mobility of these proteins on SDS polyacrylamide gels is retarded by the amount expected (roughly 11 residues for HVDAC2 and 26 residues for HVDAC2') relative to HVDAC1 (Figs. 10, 14 and 15). Since the N terminus of these and essentially all other VDAC proteins is blocked [eg.,(Kayser, Kratzin et al. 1989; Schmid, Krömer et al. 1992)], direct determination of the N terminal sequence of each protein is not possible. Results obtained with HVDAC2' expressed in COS7 cells are informative in this regard. Translation of cDNAs encoding HVDAC2' *in vitro* results in the production of two proteins that apparently differ by the alternate use of N terminal methionines for the initiation of protein synthesis; the first ATG codon present in cDNAs for HVDAC2' and an

internal ATG corresponding to the translational initiation point for HVDAC1 (Ha, Hajek et al. 1993). Since the size of the HVDAC2' protein expressed in yeast is larger than either HVDAC1 and HVDAC2, this first ATG codon is apparently used exclusively for translational initiation in yeast. However in COS7 cells, both start sites are apparently used with roughly equal frequency although it is also possible that this N terminal extension may be proteolytically cleaved when expressed in these cells. For HVDAC2, no evidence suggested that both ATG sites would be used in translation. However, HVDAC2 expressed in both yeast and COS7 cells migrated more slowly than HVDAC1 on SDS polyacrylamide gels, indicating the usage of the first ATG site by the translation machinery of both yeast and mammalian cells.

The location and nature of protein sequence motifs responsible for directing mammalian components of the mitochondria to this organelle remain to be defined. However, the fact that each of the HVDAC molecules examined in this study can be efficiently, and, it appears, exclusively targeted to mitochondria in both yeast and mammalian cells suggests that these targeting motifs are conserved and effectively recognized by the targeting machinery in both organisms. In lower eukaryotes like yeast and Neurospora, proteins of the outer mitochondrial membrane lack cleavable N terminal signal sequences present in precursors of proteins targeted to the mitochondrial matrix, inner membrane or intermembrane space (Horwich, Cheng et al. 1991; Hannavy, Rospert et al. 1993). In cases where targeting sequences have been identified for mitochondrial outer membrane proteins, the targeting sequences have been found at the amino terminus (Horwich, Cheng et al. 1991; Hannavy, Rospert et al. 1993). These N terminal targeting domains lack apparent homology and appear not to share conserved

structural features. Since each HVDAC expressed in yeast is targeted to mitochondria, it is reasonable to assume that the distinct N terminal domains present in each HVDAC directs these molecules to the mitochondria in both yeast and mammalian cells, although this remains to be demonstrated directly.

HVDAC2' molecules generated by initiation at the second ATG site or by proteolysis of N terminal extensions are essentially identical to HVDAC1 in size and N terminal sequence and might be expected to be targeted primarily to mitochondria as has been shown here for HVDAC1. However, since molecules containing the HVDAC2' specific N terminal extension (initiation at the first ATG) are equally abundant, both N termini are apparently sufficient for targeting to the mitochondria since all the epitope marker in cells transfected with tagged HVDAC2' constructs is exclusively associated with mitochondria. Thus, it may be that a diverse set of sequences lacking in apparent sequence or structural homology are responsible for directing proteins to the mammalian outer mitochondrial membrane. Alternatively, mitochondrial targeting could be directed by sequences present at the N terminus of HVDAC1 and equivalent positions in HVDAC2 and HVDAC2', since these regions are essentially identical in each of the three proteins. It is also possible that other domains within these highly homologous proteins are responsible for targeting. In this event, N terminal extensions present in HVDAC2 and HVDAC2' may function in processes unrelated to mitochondrial targeting.

It is possible that the expression systems used in this study result in overexpression of epitope-tagged forms of HVDACs relative to the endogenous, unmodified proteins. In this event, one might have expected a portion of the tagged forms of these molecules to be mislocalized to a variety

of cellular locations due to saturation of the normal mitochondrial targeting machinery. However, in each case, plasmid encoded molecules are exclusively located in mitochondria and not other cellular compartments. This suggests either that these molecules are not overexpressed in transfected cells or that if overexpressed, the machinery responsible for targeting VDAC to appropriate cellular compartments has not been saturated in these cells.

It is also formally possible that the addition of sequences encoding C terminal epitopes interferes in some way with normal targeting of one or more of the HVDAC molecules to the plasma membrane. In this event, targeting to the mitochondria might represent a "default" pathway once plasma membrane targeting has been eliminated by inclusion of epitope sequences. If C terminal epitopes interfere with plasma membrane targeting, this interference must be independent of amino acid sequence since molecules containing either of two very different amino acid sequences, the FLAG or HA epitopes, are targeted only to mitochondria.

The results presented here call into question previous reports of the presence of HVDAC1 in the plasma membrane of mammalian cells. In general, three lines of experimentation have been used to demonstrate the presence of these molecules in this cellular compartment. HVDAC1 has been observed to "co-purify" with proteins normally found in the plasma membrane, like the $GABA_A$ receptor, or with plasma membrane specialization such as caveolae (Kayser, Kratzin et al. 1989; Bureau, Khrestchatisky et al. 1992; Dermietzel, Hwang et al. 1994; Lisanti, Scherer et al. 1994). 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 non-specifically 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, Khrestchatisky et al. 1992). Second, 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, Walter et al. 1991; König, Götz et al. 1991; Cole, Adil Awna et al. 1992; Dermietzel, Hwang et al. 1994)]. Initial reports suggested that these antibodies specifically recognize plasma membrane VDAC forms (Babel, Walter et al. 1991) , although more recent studies indicate that they also react quite well with mitochondria (Konstantinova, Mannella et al. 1995). In these cases, it is easy to imagine that non-specific reactivity with plasma membranes may have been mistaken for the presence of HVDAC molecules in this cellular compartment. As shown in Figure 17, antibodies for epitopes not found in nature (FLAG) can label plasma membranes in specific cell types, although this labeling is clearly non-specific. Consistent with this interpretation, these monoclonal antibodies are only able to recognize "plasma membrane" forms of VDAC if they are added to living cells prior to fixation (Cole, Adil Awna 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, Andersson et al. 1984; Müller, Korndörfer 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 here, our results indicate that it is unlikely that any significant fraction of VDAC can be in the plasma membrane and that the vast majority of this protein is located in

mitochondria, regardless of isoform. Finally, a number of studies report the presence of plasma membrane channels with physiological characteristics similar to purified VDAC when incorporated into planar lipid bilayers (Blatz and Magleby 1983; Jalonen, Johansson et al. 1989; Dermietzel, Hwang et al. 1994). In the majority of these studies, VDAC-like channels are only observed following incubation of excised plasma membrane patches in rather unusual ionic conditions for extended periods of time (many minutes). The results presented here demonstrate that if the VDAC-like channels identified in these patch clamping studies exist in the plasma membrane under normal conditions, they are unlikely to be due to the presence of HVDAC1, as reported, or HVDAC2 or HVDAC2'. Rather, these channels must be composed of proteins that remain to be identified or characterized.

Immunoelectron microscopic examination of the distribution of HVDAC isoforms indicates that these proteins are not uniformly distributed over the surface of the mitochondria but are located within discrete patches. This would be consistent with the proposal (Kottke, Adam et al. 1988; Brdiczka 1990), and recent experimental evidence (Konstantinova, Mannella et al. 1995), that VDAC is concentrated at contact sites between the inner and outer mitochondrial membranes. Unfortunately, the resolution of the images produced in frozen thin sections was not sufficient to directly identify contact sites as the points of antibody binding in this study.

Previous studies have indicated that essentially all cell types and tissues express both HVDAC1 and HVDAC2 (Blachly-Dyson, Zambronicz et al. 1993). We demonstrate here that both isoforms are directed to mitochondria. In addition, an individual mitochondrion can contain two isoforms of VDAC since in cells expressing both HVDAC1 and HVDAC2, all mitochondria apparently contain both isoforms. At the E.M. level, these two

different isoforms appear to be present within the same restricted patch. Although the co-existence of two isoforms in the same mitochondria may be an artifact of the expression systems used in this study, these results raise the possibility that in normal mammalian cells individual mitochondria contain a mixture of VDAC isoforms. Direct demonstration of this possibility awaits the generation of antibodies which will specifically distinguish each wild-type protein. The only known functional difference between HVDAC1 and HVDAC2 is that only HVDAC1 can bind hexokinase, although sequence differences in specific domains suggest that other biochemical differences may exist (Blachly-Dyson, Zambronicz et al. 1993). Since the association of hexokinase with VDAC is dynamic and regulated, co-localization of these two isoforms within the same mitochondrial domain suggests that mitochondria can draw on pools of each molecule to establish functionally distinct complexes, perhaps at contact sites and perhaps composed of different spectra of proteins, depending on the requirements of the cell. The nature of these complexes and their functional differences await further experimentation.

To extend these studies, it would be important to test directly the possibility that insertion of an epitope at the C-terminus of an HVDAC molecule interferes with targeting to cellular compartments other than the mitochondria. To address this issue, epitopes can be placed at other positions in the primary amino acid sequence of an HVDAC molecule, preferably in putative loop regions. Expression in yeast of molecules that have been tagged in this way can be used to determine if these changes have altered the function of resulting proteins: i.e., transformants will be streaked on plates containing glycerol as the sole carbon source and growth at 30° C and 37° C. Following expression in yeast, modifed VDAC proteins can be purified and channel activity examined by reconstitution into planar lipid bilayers. If

VDAC proteins containing epitope tags at internal sites form functional channels and complement the temperature sensitivity of *vdac-* yeast strains, mammalian cells can be transfected with these constructs and the subcellular location of each protein determined by each of the three techniques used here.

Two additional HVDAC genes, HVDAC3 and HVDAC4, have been identified although little is known about their stucture or expression. It is possible that these genes encode proteins that reside in cellular compartments other than the mitochondria. To extend the analysis of these proteins, each gene can be identified in human gemonic libraries made exclusively from individual human chromosomes or regions of a chromosome containing each gene (e.g., HVDAC3, chromosome 12; HVDAC4, chromsome 1). Ultimately, cDNA for each would then be tagged with epitopes, function assessed in yeast and bilayers, constructs introduced into cultured cells and the localization of each protein examined using the systems developed in this study.

Another possible extension of the work described here would be to assess the in *vivo* function of each HVDAC isoform. One way to begin this analysis would be to create cell lines in which the expression of an individual isoform is eliminated. For example, since the HVDAC1 gene has been mapped to the X-chromosome, it may be possible to eliminate the expression of HVDAC1 in a male cell line. A diploid, immortalized human male fibroblast cell line, HT1080, has been used to successfully create lines in which the expression of another X linked gene, the human HPRT gene has been "knocked out" (Zheng, Hasty et al. 1991). The single HVDAC1 gene present in the HT1080 cell line could be eliminated by similar homologous recombination approaches, creating cell lines in which the functions of HVDAC1 could then studied. "Double knock-out" strategies also exist which

could be used to create cell lines in which expression of individual HVDAC genes residing on autosomes is eliminated. This set of cell lines would be invaluable tools for assessing the *in vivo* function of each HVDAC gene and for addressing the question of why higher organisms have evolved a family of these genes.

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