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# Mutation of K234 and K236 in the Voltage-Dependent Anion Channel 1 Impairs Its Insertion into the Mitochondrial Outer Membrane

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Previous *in vitro* studies indicated that mutation of both K234 and K236 to arginine, glutamine, or glutamic acid impaired the ability of the voltage-dependent anion channel (VDAC1) to insert into the outer membrane of the mitochondria (Smith *et al.* 1995). These same mutants were expressed in a strain of *Saccharomyces cerevisiae* with a disruption in the VDAC1 gene. The mutant VDAC1 forms were found in the mitochondria suggesting that they were correctly sorted to the outer membrane. However, only very small amounts of the mutants were inserted into the mitochondrial membranes. Mitochondria isolated from the strains expressing the mutants were capable of catalyzing the translocation of both wild-type VDAC1 and pre-alcohol dehydrogenase III indicating that the translocation apparatus was functional. These results confirm the previously drawn conclusion that K234 and K236 are part of a membrane insertion motif. The failure of the mutant VDAC1 forms to insert did not cause VDAC1 precursors to accumulate in the soluble cell cytoplasm or in the microsomal fraction. The apparent lack of a "precursor pool" suggested that a post-transcriptional control mechanism might limit the amounts of VDAC1 precursors in the cell. Such a control mechanism is consistent with the observation that the amount of VDAC1 was very similar after epichromosomal (gene in a 2u plasmid controlled by a Gal1 promoter) and chromosomal expression (endogenous gene controlled by the endogenous promoter).

**KEY WORDS:** VDAC1; mitochondria.

## INTRODUCTION

The voltage-dependent union channel of the mitochondrial outer membrane (VDAC1 or mitochondrial porin) sits at a critical junction between the extramitochondrial cytoplasm and the inner compartments of the mitochondrion. VDAC1 forms large pores that can be shown to open and close in response to applied potentials and a region of these proteins that is sensitive to voltage changes has been identified (Thomas *et al.*, 1993). The principal metabolic function of the VDAC1 seems to be the formation of channels that allow passage of large, charged biochemicals and to regulate their exchange across the outer membrane (OM) (Rostovtseva and Colombini, 1997). These biochemicals are transported by inner membrane carriers into the matrix of the mitochondrion as substrates for oxidative phosphorylation and other reactions.

Like other outer membrane proteins, VDACs are transcribed from nuclear genes and translated on cytoplasmic ribosomes. In yeast, VDAC1 can insert into the OM either cotranslationally (Fujiki and Verner, 1991) or by a post-translational process that may involve chaperones (Komiya *et al.*, 1996). The post-translational insertion reaction requires ATP (Kleene *et al.*, 1987) and is catalyzed by a complex of outer membrane proteins that includes Tom70, Tom 40, Tom22, Tom20, Tom7, and Tom6 (Kunkele *et al.*, 1998). During the initial steps of post-translational

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membrane insertion, VDAC1 may recognized by Tom70 and Tom20, two members of a complex of outer membrane receptor proteins (Komiya *et al.*, 1997).

The molecular signals in VDAC1 that are recognized by the outer membrane complex (i.e., sorting signals) are not known. However, *in vitro* experiments with a mutant in which the C-terminal 15 amino acids in VDAC1 were deleted suggested that this tract might contain a sorting signal (Court *et al.*, 1996). Another C-terminal signal that appears to be involved in membrane insertion has been identified by *in vitro* experimentation. Two lysyl residues, K234 and K236 were shown to be crucial for the membrane insertion of yeast VDAC1 (Smith *et al.*, 1995). Point mutation of these residues to arginine, glutamine, or glutamate markedly impaired insertion of VDAC1 into the outer membrane. In experiments described here, yeast VDAC1 was mutated at K234 and K236 and expressed in an auxotrophic strain of *S. cerevisiae*. These experiments were performed to determine whether these two lysines had an effect on membrane insertion under physiological conditions.

## MATERIALS AND METHODS

### The Construction of Wild-Type VDAC1 and Mutant VDAC1 Expression Vectors and Their Transformation into *Saccharomyces cerevisiae*

The construction of the three mutant VDAC1 forms (K234R; K236R VDAC1, K234Q; K236Q VDAC1, and K234E; K236E VDAC1) has been previously described (Smith *et al.*, 1995). Each of the mutants and the wild-type VDAC1 were subcloned into the *Sacl* and *Xbal* sites in the multiple cloning region of the 2 μ multicopy *in vivo* expression vector, pYes2.0 (Stratagene, CA). In these constructs, the expression of the VDAC1 forms was under the control of the GAL1 promoter.

The various pYes2.0 constructs and unmodified pYes2.0 were transformed into the JHRY20-2Ca of *S. cerevisiae* in which the VDAC1 gene had been disrupted by integration of the LEU2 marker (*dp1*). In addition, JHRY20-2Ca with the VDAC1 gene intact (*wt*) was transformed with unmodified pYes2.0. The lithium carbonate procedure using commercially prepared reagents (Bio 101, CA) was employed for this purpose. Both *wt* and *dp1* were auxotrophic for uracil (see Table I). pYes2.0 carries the URA3 gene and transformants were selected on agar plates containing 2% glucose in yeast nitrogen base supplemented with essential nutrients except uracil. Transformants were grown in liquid culture (2% glucose in yeast nitrogen base supplemented with essential nutrients except uracil) to an absorbance of about 1.0 at 600 nm and stored for up to 2 weeks at 4°C before use.

### Preparation of Spheroplasts and Mitochondria

In yeast nitrogen base supplemented with essential nutrients except uracil 250 ml of 2% glucose was inoculated at a density of $10^7$ cells/ml and incubated for 4 h at 30°C with shaking at 300 rpm. The cells were collected by centrifugation at 1500 × *g* for 5 min, washed with water, and incubated in the same medium with 2% galactose and 1% raffinose instead of glucose for 4 h more. The cells were collected by centrifugation, resuspended, and washed once with 50 ml of distilled water. They were resuspended in 20 ml 0.1 M Tris–sulfate (pH = 9.4)–1 mM dithiothreitol (DTT) and incubated at 30°C for 15 min. The cells were again collected by centrifugation, resuspended, and washed once in 20 ml of 1.2 M sorbitol–25 mM $KP_i$ (pH = 7.5). They were resuspended in 20 ml of the same sorbitol–$KP_i$ mixture containing 1 mg/ml Zymolyase 20T (ICN, CA) and incubated for 30 min at 30°C. The spheroplasts were collected by centrifugation at 4°C and washed without resuspension in 20 ml ice cold sorbitol–$KP_i$. Finally, they were gently resuspended in 20 ml ice cold 1.2 M sorbitol–1 mM phenylmethylsulfonyl fluoride–20 mM HEPES (pH = 7.5). The spheroplasts were either used directly or used to prepare mitochondria, as has been described

**Table I.** *Saccharomyces cerevisiae* Strains[a]

| |
|---|
| *wt* JHRY20-2Ca: MATa, *GAL*+, leu2, his3-Δ200 and ura3-52 |
| *wt/y2* wt transformed with pYes2.0 |
| *dp1* JHRY20-Ca: MATa, *GAL*+, yvdac1::LEU2, his3-Δ200 and ura3-52 |
| *dp1/y2* dp1 transformed with pYes2.0 |
| *dp1/wt* dp1 transformed with pYes2.0–VDAC1 |
| *dp1/2r* dp1 transformed with pYes2.0–K234R, K346R VDAC1 |
| *dp1/2q* dp1 transformed with pYes2.0–K234Q, K346Q VDAC1 |
| *dp1/2e* dp1 transformed with pYes2.0–K234E, K346E VDAC1 |

[a] The abbreviation for each strain used in this study is given in italics. The name, genotype, and vectors associated with the strain are in normal type.

previously (Smith *et al.*, 1994). Protein concentrations for either spheroplasts or mitochondria were determined using commercially prepared bicinchoninic acid reagent and cupric sulfate according to the manufacturer's instructions (Sigma, MO).

### Other Methods

The methods for analysis of *in vitro* protein translocation into mitochondria, immune blotting procedures, and electrophoretic methods have been described elsewhere (Smith *et al.*, 1994). However, the procedure used to prepare samples prior to immune blotting was modified. Aliquots containing spheroplasts or mitochondria were added to 2 volumes of ice cold 10% trichloroacetic acid and held on ice for 10 min. The precipitate was collected by centrifugation for 10 min at 14,000 $\times$ *g*. The trichloroacetic acid precipitation was essential in order to avoid partial degradation of VDAC1 and its mutants that occurred during solubilization prior to electrophoresis. The protein precipitate was resuspended in 25 μl of 1 M Tris. Then, 25 μl of a buffer containing 2% sodium dodecyl sulfate, 2% mercaptoethanol, 40% glycerol, and 0.2% bromphenol blue in 100 mM Tris–chloride (pH = 8.3) was added. The samples were incubated for 10 min at 65°C prior to electrophoresis. Total RNA was extracted from yeast using a method described by others (Kohrer and Domdey, 1991). Northern blotting was performed using a commercial kit (Ambion, TX).

### Materials

Reagents not mentioned above were obtained in the highest possible purity from various manufacturers. The antibody used for detection of VDAC1 and its mutant forms was directed against the first 19 amino acids of wild-type VDAC1 (Blachly-Dyson *et al.*, 1997). This antibody and the yeast strains employed here were the gifts of M. Forte.

## RESULTS

### Both Exogenously and Endogenously Expressed VDAC1 Insert into Mitochondrial Membranes to a Similar Extent

An auxotrophic strain of *S. cerevisiae* that is wild type for VDAC1 (JHRY20-2Ca) was transformed with the 2u plasmid, pYes2.0, to produce *wt/y2*. This strain and other strains used here are described more fully in Table I. A version of the JHRY20-2Ca strain in which the VDAC1 gene had been disrupted (dp1) was transformed with pYes2.0 or pYes2.0 containing the VDAC1 gene under the control of a Gal1 promoter. These strains were named *dp1/y2* and *dp1/wt*, respectively. The three strains were grown to mid-log phase on glucose and then shifted to galactose/raffinose. After 8 and 16 h of growth, spheroplasts were prepared and analyzed for their content of VDAC1 by immune blotting. As expected, VDAC1 was not detectable in spheroplasts prepared from the *dp1/y2* strain (not shown). However, the *wt/y2* (endogenous expression) and *dp1/wt* (expression from a 2u plasmid) strains expressed about the same amount of VDAC1 after both 8 and 16 h of growth in the galactose-containing medium (Fig 1).

### Mutations at K234 and K236 Reduce the Ability of VDAC1 to Insert into Mitochondrial Membranes

Mutation of K234 and K236 has been shown to impair the ability of VDAC1 to insert *in vitro* into the outer membrane of yeast mitochondria (Smith *et al.*, 1995). To determine whether this effect could be reproduced *in vivo*, the VDAC1 disrupted JHRY20-2Ca strain, *dp1*, was transformed with pYes2.0 containing each of three mutant VDAC1 forms: K234R; K236R VDAC1, K234Q; K236Q VDAC1, and K234E;

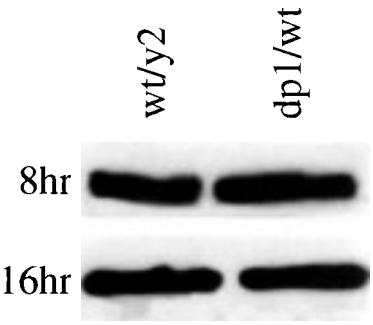


**Fig. 1.** Comparison of chromosomal and epichromosomal expression of VDAC1. The *dp1/wt* and *wt/y2* strains were preincubated at an initial cell density of $10^5$ cells/ml in minimal medium minus uracil with 2% glucose for 4 h. The carbon source was changed to 2% galactose and 1% raffinose. At 8 and 16 h after the shift in carbon source, cells were harvested and spheroplasts were prepared. Spheroplast protein (100 μg) was analyzed for its VDAC1 content by electrophoresis and immune blotting. This experiment is typical of three.

K236E VDAC1. In each of these strains (*dp1/2r, dp1/2q*, and *dp1/2e*, respectively), the mutant VDAC1 gene was under the control of a Gal1 promoter. The three strains expressing mutant VDAC1 forms as well as *dp1/y2* and *dp1/wt* were grown to mid-log phase on glucose, shifted to galactose/raffinose, and incubated for 4 h Spheroplasts were isolated and mitochondria, microsomes, and soluble cytoplasm were prepared from each of the strains. Each of these fractions was analyzed by immune blotting for its VDAC1 content using an antibody that only recognized the extreme N terminus of VDAC1. This antibody would be expected to react with all of the VDAC1 mutants; however, it would not recognize VDAC1 fragments that did not contain the N terminus. In fact, VDAC1 fragments were not detected in any of the immune blots. The mitochondria were also analyzed with antibodies against the β subunit of the $F_1$-ATPase (β-$F_1$).

Mitochondria from all five strains contained roughly equivalent amounts of β-$F_1$ (Fig. 2). As was expected, VDAC1 could not be detected in mitochondria from *dp1/y2*, but was obvious in mitochondria from *dp1/wt*, the strain expressing wild-type VDAC1. The mitochondria from the strains expressing mutant VDAC1 forms contained only trace amounts of VDAC1. The lower amount of mutant VDAC1 forms in mitochondria was paralleled by lower amounts of VDAC1 in intact spheroplasts (Fig. 3A). Neither VDAC1 nor the mutant forms were detected in either the soluble cytoplasm or microsomal fractions from any of the strains (not shown). These observations indicate that VDAC1 and its mutant forms accumulated predominantly, perhaps exclusively, in the mitochondria. However, the presence of very small amounts in other cellular compartments was not ruled out.

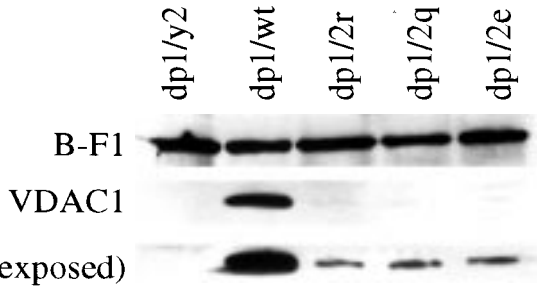


**Fig. 2.** Comparison of the *in vivo* membrane insertion of wild-type and mutant VDAC 1. Each of five strains, *dp1/y2, dp1/wt, dp1/2r, dp1/2q*, and *dp1/2e* were preincubated at an initial cell density of $10^5$ cells/ml in minimal medium minus uracil with 2% glucose for 4 h. The carbon source was changed to 2% galactose and 1% raffinose. At 4 h after the shift in carbon source, cells were harvested and mitochondria were prepared. Mitochondrial protein (25 μg) was analyzed for its content of both the β subunit of the $F_1$ ATPase (β-$F_1$) and VDAC1 by SDS-PAGE and immune blotting. This experiment is typical of three.

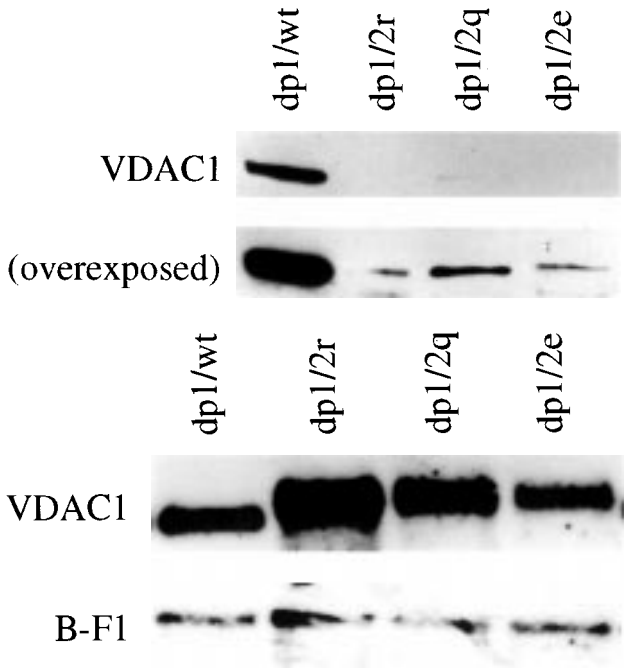


**Fig. 3.** Comparison of the amounts of VDAC1 and epichromosomal VDAC1 transcripts in cells expressing wild-type and mutant VDAC1. Each of four strains, *dp1/wt, dp1/2r, dp1/2q*, and *dp1/2e* were preincubated at an initial cell density of $10^5$ cells/ml in minimal medium minus uracil with 2% glucose for 4 h. The carbon source was changed to 2% galactose and 1% raffinose and the cultures were incubated for an additional 4 h. (A) Cells were harvested and spheroplasts were prepared. Spheroplast protein (100 μg) was analyzed for its VDAC1 content by electrophoresis and immune blotting. This experiment is typical of three. (B) Cells from 100 ml of each culture were extracted for total RNA. Approximately 25 μg of RNA was analyzed for its content of transcripts of both VDAC1 and the β subunit of the $F_1$ ATPase (β-$F_1$). This experiment is one of two similar determinations.

The amounts of the VDAC1 transcripts (relative to β-$F_1$ mRNA) in intact spheroplasts were equivalent (Fig. 3B). Furthermore, the amount of VDAC1 mRNA always exceeded the amount of β-$F_1$ mRNA. Since the mitochondrial content of β-$F_1$ was relatively constant among the strains (Fig. 2), it is unlikely that the differences in amounts of cellular or mitochondrial VDAC1 can be explained by differences in transcription. In addition, *d1/y2p, dp1/wt*, and the three strains expressing the mutant VDAC1 forms were all capable of incorporation of radiolabeled leucine into protein (Fig. 4). The differences in VDAC1 content cannot be explained by a general failure of protein synthesis in the strains expressing mutant VDAC1 forms.

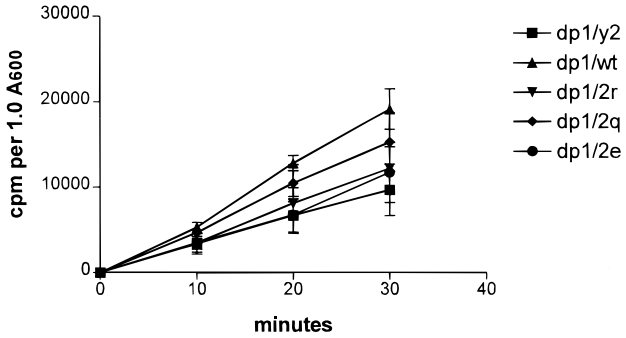


**Fig. 4.** Comparison of the rates of protein synthesis in cells expressing wild-type and mutant VDAC1. Each of five strains, *dp1/y2*, *dp1/wt*, *dp1/2r*, dp1/2q, and *dp1/2e* were preincubated at an initial cell density of $10^5$ cells/ml in minimal medium minus uracil with 2% glucose for 4 h. The carbon source was changed to 2% galactose and 1% raffinose. At 4 h after the shift in carbon source, cells were collected by centrifugation and resuspended to a concentration of 1.0 $A_{600}$ per ml in minimal medium minus leucine with 2% galactose and 1% raffinose. [$^3$H] leucine was added to a final concentration of 2μCi/ml. The amount of radioactivity incorporated into the trichloracetic acid insoluble fraction was estimated at the indicated intervals. Each point is the average (+/− standard deviation) of three to five determinations.

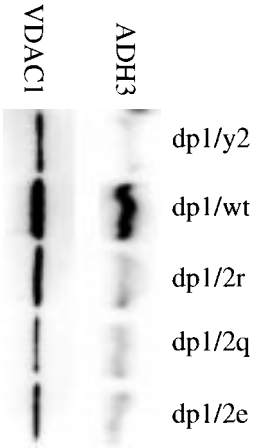


**Fig. 5.** Comparison of the *in vitro* translocation of pre-alcohol dehydrogenase III (pADH3) and VDAC1 into mitochondria isolated from cells expressing wild-type and mutant VDAC1. Each of five strains, *dp1/y2*, *dp1/wt*, *dp1/2r*, *dp1/2q*, and *dp1/2e* were preincubated at an initial cell density of $10^5$ cells/ml in minimal medium minus uracil with 2% glucose for 4 h. The carbon source was changed to 2% galactose and 1% raffinose. At 4 h after the shift in carbon source, cells were harvested and mitochondria were prepared. Mitochondrial protein (200 μg) per ml were incubated with *in vitro* synthesized and radiolabeled pADH3 or VDAC1 in the presence of an ATP generating system. After 30 min at 30°C, the samples were removed to ice and treated for 20 min with 100 μg/ml of proteinase k. Mitochondria were isolated by centrifugation and analyzed for their content of radiolabeled mature ADH3 and VDAC1 by electrophoresis and fluorography.

### Mitochondria Isolated from *Saccharomyces cerevisiae* that Express Mutant VDAC1 Forms are Capable of the Translocation of Wild-Type VDAC1 and Pre-Alcohol Dehydrogenase III

It was clear that mutation of both K234 and K236 impaired the *in vivo* insertion of VDAC1 into mitochondrial membranes. It was not obvious, however, whether this effect was because the mitochondrial translocation apparatus was defective in the strains expressing the mutants or because the mutant VDAC1 forms were unable to undergo the translocation reaction. After 4 h of growth in galactose/raffinose, mitochondria were prepared from *dp1/y2*, *dp1/wt*, and the three strains expressing mutant VDAC1 forms. These mitochondria were analyzed for their ability to insert VDAC1 into the outer membrane and to import pre-alcohol dehydrogenase III (pADH3) into the matrix (Fig. 5). In all of the strains, the *in vitro* translocation of both precursors was ATP dependent while mitochondrial accumulation of ADH3, but not VDAC1, was abolished by valinomycin (data not shown).

Mitochondria isolated from the strain expressing wild-type VDAC1 (*dp1/wt*) were able to import both VDAC1 and pADH3 *in vitro* more efficiently than mitochondria isolated from *dp1/y2*. That is, expression of wild-type VDAC1 appeared to complement the inefficient import by the VDAC1 knockout. The mitochondria isolated from strains expressing mutant forms of VDAC1 also imported pADH3 more efficiently than mitochondria isolated from *dp1/y2*. Mitochondria isolated from the strains expressing the three VDAC1 mutants catalyzed the membrane insertion of VDAC1 about as efficiently as mitochondria from *dp1/y2*. It was clear that mitochondria isolated from each of the strains expressing mutant forms of VDAC1 were at least as capable as those isolated from *dp1/y2*. Differences in the ability of mitochondria to insert/import precursors could not have accounted for the extreme differences in the amounts of mutant VDAC1 forms that accumulated in the mitochondria *in vivo* (Fig. 2).

### Discussion

Previous *in vitro* studies indicated that mutation of both K234 and K236 to arginine, glutamine, or glutamic acid impaired the ability of VDAC1 to insert into the outer membrane of the mitochondria (Smith *et al.*, 1995). These same mutants were expressed in

a strain of *S. cerevisiae* with a disruption in the VDAC1 gene. Only trace amounts of the mutant VDAC1 forms were found in the mitochondria, indicating that the mutations impaired the insertion of VDAC1 into the outer membrane *in vivo* as well as *in vitro*. The manner in which these mutations affected VDAC1 insertion was not clear. The possibility that the mutants blocked their own membrane insertion and, perhaps, the import of other mitochondrial precursors by inactivating the import apparatus was considered. This does not seem to be the case, since mitochondria isolated from the mutant strains were capable of catalyzing the translocation of both wild-type VDAC1 and pADH3 at least as well as mitochondria from the VDAC1 knockout. Apparently, K234 and K236 are part of a motif that is crucial for the membrane insertion of VDAC1 both *in vivo* and *in vitro*.

The expression of wild, type VDAC1 complemented the normally slow translocation of both pADH3 and VDAC1 in the VDAC1-disrupted strain. This was surprising since recent experiments have shown that VDAC1 is apparently not an essential component of the outer membrane import apparatus (Kunkele *et al.*, 1998). Since VDAC1 is involved in the passage of ATP across the outer membrane (Rostovtseva and Colombini, 1997), a VDAC1 deficiency might impair translocation by restricting ATP movement. However, the effects of disruption of the VDAC1 gene on yeast mitochondria include deficiencies in components of the electron transport chain (Dihanich *et al.*, 1987). These or other undescribed deficiencies may account for the impaired protein translocation.

Although the membrane insertion of the mutant VDAC1 forms was impaired, the amounts of mutant forms in whole spheroplasts were low. Neither mutant nor wild-type VDAC1 could be detected in the soluble cytoplasm or the microsomal fraction. That is, there was no evidence for precursor pools, such as have been described when mitochondrial translocations are inhibited (Yaffe and Schatz, 1984). The absence of these pools could not be explained by differences in the efficiency of transcription in strains expressing the mutant VDAC1 forms. It is more likely that the amount of VDAC1 precursor is controlled by a post-transcriptional mechanism. This conclusion is consistent with the observation that the amount of wild-type VDAC1 in the cell was roughly the same whether the gene was controlled by endogenous promoters or by a Gal1 promoter on a 2u plasmid. The mechanism of this putative post-translational control is not clear. Failure to identify fragments of the various VDAC1 forms does not preclude control by proteolysis since the antibody would not detect any fragment that had lost its N terminus. Robust rates of protein synthesis do not rule out a selective inhibition of VDAC1 synthesis by accumulated precursor or precursor fragments. In any case, it would not be surprising if the amount of precursor VDAC1 was under tight control. The VDAC1 precursor can assume conformations that allow it to insert into synthetic and natural membranes spontaneously (Keene *et al.*, 1987). If such a thing were to happen in the cellular cytoplasm, the formation of inappropriate pores could have serious consequences for the cell.

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