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Multicopy Suppressors of Phenotypes Resulting from the Absence of Yeast VDAC Encode a VDAC-Like Protein

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The permeability of the outer mitochondrial membrane to most metabolites is believed to be based in an outer membrane, channel-forming protein known as VDAC (voltage-dependent anion channel). Although multiple isoforms of VDAC have been identified in multicellular organisms, the yeast *Saccharomyces cerevisiae* has been thought to contain a single VDAC gene, designated *POR1*. However, cells missing the *POR1* gene (Δ*por1*) were able to grow on yeast media containing a nonfermentable carbon source (glycerol) but not on such media at elevated temperature (37°C). If VDAC normally provides the pathway for metabolites to pass through the outer membrane, some other protein(s) must be able to partially substitute for that function. To identify proteins that could functionally substitute for *POR1*, we have screened a yeast genomic library for genes which, when overexpressed, can correct the growth defect of Δ*por1* yeast grown on glycerol at 37°C. This screen identified a second yeast VDAC gene, *POR2*, encoding a protein (YVDAC2) with 49% amino acid sequence identity to the previously identified yeast VDAC protein (YVDAC1). YVDAC2 can functionally complement defects present in Δ*por1* strains only when it is overexpressed. Deletion of the *POR2* gene alone had no detectable phenotype, while yeasts with deletions of both the *POR1* and *POR2* genes were viable and able to grow on glycerol at 30°C, albeit more slowly than Δ*por1* single mutants. Like Δ*por1* single mutants, they could not grow on glycerol at 37°C. Subcellular fractionation studies with antibodies which distinguish YVDAC1 and YVDAC2 indicate that YVDAC2 is normally present in the outer mitochondrial membrane. However, no YVDAC2 channels were detected electrophysiologically in reconstituted systems. Therefore, mitochondrial membranes made from wild-type cells, Δ*por1* cells, Δ*por1* Δ*por2* cells, and Δ*por1* cells overexpressing YVDAC2 were incorporated into liposomes and the permeability of resulting liposomes to nonelectrolytes of different sizes was determined. The results indicate that YVDAC2 does not confer any additional permeability to these liposomes, suggesting that it may not normally form a channel. In contrast, when the VDAC gene from *Drosophila melanogaster* was expressed in Δ*por1* yeast cells, VDAC-like channels could be detected in the mitochondria by both bilayer and liposome techniques, yet the cells failed to grow on glycerol at 37°C. Thus, channel-forming activity does not seem to be either necessary or sufficient to restore growth on nonfermentable carbon sources, indicating that VDAC mediates cellular functions that do not depend on the ability to form channels.

To synthesize ATP, mitochondria must transport a wide variety of charged solutes (e.g., phosphate, pyruvate, ATP, and ADP) across both the inner and outer membranes that form this organelle. The two membranes accomplish this transport by different mechanisms. The inner membrane uses specific transporters and permeases to move these compounds in and out of the matrix. In contrast, permeation through the outer membrane is believed to be based in an outer membrane protein called VDAC (also known as mitochondrial porin), which forms a voltage-dependent, anion-selective channel. Purified VDAC protein, when introduced into phospholipid membranes, forms aqueous pores large enough for passage of metabolic intermediates such as ATP (23). These pores close in response to applied voltage, and this closure can be modulated by application of NADH (30), by a protein found in the mitochondrial intermembrane space (16), and by synthetic polyanions (7). Closure of the VDAC channel is associated with a change in the permeability of mitochondria to relevant metabolites (2, 6). VDAC protein is also the binding site for several kinases, such as hexokinase and glycerol kinase, which bind reversibly to the mitochondrial outer membrane. This binding varies with tissue type and developmental stage and is particularly pronounced in some highly glycolytic tumor cells (reviewed in reference 1). Binding to the outer membrane is thought to give these enzymes preferential access to ATP produced by the mitochondria and, conversely, to give the mitochondria preferential access to the ADP produced by these enzymes. These results are consistent with the idea that VDAC provides the primary permeability pathway for relevant metabolites across the outer membrane and thus may constitute a site at which mitochondrial function can be regulated through regulation of metabolite traffic across this membrane. In addition, there may exist cellular roles for VDAC that do not require channel function, for example, as a binding site for a variety of metabolic enzymes.

Although multiple isoforms of VDAC have been identified in mammals (4, 14, 26) and plants (9, 15), the yeast *Saccharomyces cerevisiae* has been thought to contain a single VDAC gene (11, 21). This gene, designated *POR1* (or *OMM2*), encodes a 283-residue protein which forms channels that have been extensively characterized in synthetic phospholipid bilay-

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ers. Southern blots of yeast genomic DNA probed with the *POR1* gene at reduced stringency revealed only the expected fragments for that gene, suggesting that VDAC in yeast is encoded by a single gene (10a). Consistent with this conclusion, deletion of the *POR1* gene by homologous recombination (Δ*por1*) resulted in cells which produce no immunologically detectable VDAC protein with an antibody raised against purified yeast VDAC (8). Thus, yeast appeared to contain only a single gene encoding a VDAC protein. Since VDAC is thought to provide the primary pathway for the movement of metabolites across the outer mitochondrial membrane, it was expected that cells in which the *POR1* gene had been deleted (Δ*por1* cells) would be deficient for respiration. Surprisingly, Δ*por1* cells were able to grow on yeast media containing a nonfermentable carbon source (glycerol), although, unlike wild-type cells, they could not grow on glycerol-based media at elevated temperature (37°C) (3). If VDAC normally provides the pathway for metabolites to pass through the outer membrane (or provides any other mitochondrial function essential for respiration), some other protein(s) must be able to partially substitute for that function.

To identify proteins that could functionally substitute for VDAC, we have screened a yeast genomic library for genes which, when overexpressed by virtue of their inclusion in a multicopy plasmid, can correct the growth defect of Δ*por1* yeast grown on glycerol at 37°C. This screen yielded a second yeast VDAC gene (*POR2*), encoding a protein (YVDAC2) with 49% amino acid sequence identity to the previously identified yeast VDAC protein (YVDAC1). Genetic and molecular characterization of the protein product of this gene indicates that channel-forming activity does not seem to be either necessary or sufficient to restore growth on nonfermentable carbon sources, leading to the conclusion that VDAC mediates cellular functions that do not depend on the ability to create channels forming a pathway for the movement of charged molecules across the outer membrane.

MATERIALS AND METHODS

Materials, media, and strains. M3 (*MAT***a** *lys2 his4 trp1 ade2 leu2 ura3*) is the parental *POR1 POR2* yeast strain used to generate Δ*por* strains. M22-2 contains a deletion of most of the *POR1* coding region by insertion of the yeast *LEU2* gene at this locus (3). M3-2 contains a deletion of most of the *POR2* coding region by insertion of the yeast *TRP1* gene (see below). M22-2-1 is the Δ*por1* Δ*por2* double mutant. The yeast media YPD (glucose as the carbon source), YPG (glycerol as the carbon source) and SMM (supplemented with adenine, uracil, tryptophan, histidine, leucine, and lysine as needed; glucose as the carbon source) were prepared as described in reference 17.

Construction and screening of a genomic library from Δ*por* cells. Genomic DNA was prepared (17) from strain M22-2, partially digested with *Sau*3AI to a size of 5 to 20 kb, and ligated into *Bam*HI-digested pSEY8, a 2μm-based *URA3* yeast shuttle vector (10). Approximately 2.1×10^5 transformants were obtained in *Escherichia coli*. Bacterial colonies were scraped off the plates in two pools and used for plasmid DNA preparation.

Yeast strain M22-2 was transformed (27) with DNA from the above library, and transformants were selected on SMM plates lacking uracil. Approximately 10^5 Ura^+ colonies were obtained (4.5×10^4 from pool A and 5.8×10^4 from pool B). The transformation plates were replica plated to YPG and grown at 37°C until colonies grew up from the background (3 to 5 days). Colonies that grew were restreaked onto YPG plates and grown at 37°C. The transformants that grew upon restreaking were used for plasmid isolation (see below), "cured" of the plasmids by extensive growth on YPD, and streaked onto YPD plates. The streakouts were replica plated to SMM lacking uracil to identify colonies that had lost the plasmid, and these were tested for growth at 37°C on YPG to test whether the ability to grow had been lost with the plasmid.

DNA was prepared from transformed yeast by inoculating individual colonies into 3 ml of SMM lacking uracil. The cells were then grown overnight at 30°C, pelleted by centrifugation, resuspended in 50 mM Tris-Cl (pH 7.5)–10 mM EDTA–0.3% β-mercaptoethanol containing 1 mg of Zymolyase 100T (Seigaku, Tokyo, Japan) per ml, and incubated at 37°C for 60 min. A 20-μl volume of 10% sodium dodecyl sulfate (SDS) was then added, followed by 100 μl of 7 M ammonium acetate, and the contents of the tubes were mixed and frozen at −80°C for 15 min. The tubes were centrifuged for 15 min in a microcentrifuge at top speed, and the supernatants were precipitated with 0.7 volume of isopropanol. After 15 min at −80°C, the samples were centrifuged for 15 min at high speed in a microcentrifuge. The pellets were washed with 70% ethanol, vacuum dried, and resuspended in 20 μl of TE (10 mM Tris-Cl [pH 7.5], 1 mM EDTA). A 5-μl volume of each preparation was used to transform *E. coli*, or 1 μl was amplified by PCR.

DNA sequencing was performed by the Vollum Institute sequencing facility. Database searches were performed at the National Center for Biotechnology Information using the BLAST network service.

Construction of *POR2* deletions. A 2.1-kb DNA fragment containing the *POR2* gene was inserted into Bluescript plasmid (pBSKM13- [Stratagene]). An 840-bp *Pst*I-*Eco*RI fragment encoding the yeast *TRP1* gene was inserted between the *Sac*I and *Stu*I sites in the *POR2* gene, deleting the initiation codon and most of the open reading frame of *POR2*. The resulting construct was excised from the vector with *Bam*HI and *Xho*I, and the linear fragment containing the disrupted *POR2* gene was gel purified. This *TRP1*-containing fragment was then used to transform yeast strains M-3 and M22-2 containing the *POR1* gene on a plasmid. Trp^+ transformants were tested for the stability of the Trp^+ phenotype by extensive growth on YPD. DNA was isolated from stable transformants and amplified with oligonucleotide primers specific for *POR2* sequences flanking the site of *TRP1* insertion (YV2-5P [5′CGCTTCCAATTGCAGAAATGG3′] and YV2-3P [5′AAACACTGGTCGTAGGTCACG3′]). Amplified fragments were subsequently analyzed by restriction digestion to assess the structure of the *POR2* locus. To generate Δ*por1* Δ*por2* double mutants, Trp^+ M22-2 transformants containing the *POR1* gene on a plasmid were "cured" of the POR1-containing plasmid by growing cells extensively in YPD, streaking on YPD, and screening for plasmid loss by replica plating to SMM plates lacking uracil.

The growth phenotypes of both Δ*por1* and Δ*por1* Δ*por2* yeast strains varied widely with the genetic background of the yeast strain. Several strains tested were able to grow on glycerol at 37°C even with the Δ*por1* mutation. On the other hand, we were unable to obtain viable Δ*por1* Δ*por2* derivatives in two strains tested. The strains described in this paper are all derived from the same background (M3) as the original Δ*por1* strain (3).

Construction of VDAC promoter switches. To place the *POR2* coding region under control of the *POR1* promoter and vice versa, *Nco*I sites were introduced into both genes at the translation initiation codons. This changed codon 2 of *POR1* from serine to alanine and had no effect on the coding capacity of *POR2*. The *POR1* and *POR2* promoters consist of sequences representing the 560 and 558 bp immediately upstream of the translational initiation codon of each gene. Both constructs were subcloned into a centromere-based yeast plasmid (pSEYC58) and into a 2μm-based yeast plasmid (pSEY8) and introduced into yeast strain M22-2 by transformation. Transformants were tested for growth on YPG plates at 37°C.

To construct yeast strain M3-YV1PYV2, a plasmid which contained the following DNA fragments from 5′ to 3′ was constructed: 618 bp of *POR1* 5′ untranslated sequence; the yeast *URA3* gene; 352 bp of additional *POR1* 5′ untranslated sequence, ending at the start codon; the *POR2* coding sequence; and 536 bp of *POR1* 3′ sequence. This fragment was released from the plasmid by restriction digestion, and used to transform strain M22-2 to Ura^+. Colonies that were Ura^+ Leu^- (indicating that the construct had substituted for the *LEU2* gene at the *POR1* locus in M22-2) were subjected to whole-cell PCR (17) with primers specific for the *POR1* 5′ and 3′ sequences, and PCR fragments of the correct size were digested with *Hpa*I to verify that they contained the *POR2* insert.

Expression of *Drosophila* VDAC in yeast. To express *Drosophila melanogaster* VDAC in yeast, the *Drosophila* VDAC coding region was inserted between the 5′ and 3′ untranslated sequences of YVDAC1 (25), the URA3 gene was inserted into the 5′ untranslated region, and the resulting construct was integrated into yeast M22-2 as with M3-YV1PYV2 above.

Antibody production and immunoblotting. Peptides representing the N termini of YVDAC1 (MSPPVYSDISRNINDLLNK) and YVDAC2 (MALRFFNDISRDVNGLFNR) were synthesized, conjugated to keyhole limpet hemocyanin, and injected into rabbits by Macromolecular Resources, Fort Collins, Colo. Sera from immunized rabbits was screened and checked for specificity on immunoblots containing mitochondrial proteins prepared from yeast strains M3, M22-2, M3-2, and M22-2-1. In all cases, positive immunoreactive bands were completely eliminated by preincubation of the appropriate dilutions of each serum overnight at 4°C with 5 μg of cognate peptide per ml. To increase the specificity of YVDAC2 sera, appropriate dilutions of sera from YVDAC2-injected rabbits were incubated overnight at 4°C with 10 μg of YVDAC1 peptide per ml. Antibody to *Drosophila* VDAC was kindly provided by J. Ryerse.

Protein fractions were separated on 10% polyacrylamide–SDS gels and transferred to nitrocellulose by electroblotting. The blots were then blocked in 5% nonfat milk and incubated with appropriate dilutions of antibody in 5% nonfat milk, and antibody binding was visualized with alkaline phosphatase-conjugated goat anti-rabbit antibodies and colorimetric substrates.

Vital staining of yeast mitochondria. To observe mitochondrial shape and distribution in live yeast cells, the cells were grown in liquid culture in YPG medium at 30°C and then shifted to 37°C for 18 to 24 h or maintained at 30°C, with the cell densities adjusted to be between 0.5 and 1.5 after the 24-h incubation. The cells were centrifuged and resuspended in ~1/10 of the original volume of YPG medium and then mixed with an equal volume of a 200-μg/ml aqueous

solution of 2-(4-dimethylaminostyryl)-1-methylpyridinium iodide (DASPMI; Molecular Probes, Eugene, Oreg.), a vital stain that is taken up specifically by mitochondria that have a membrane potential (28). To immobilize stained cells, the cell-dye suspension was mixed with an equal volume of 2% low-melting-point agarose at 42°C and placed on a prewarmed slide before adding a coverslip. The cells were viewed within 15 min under a fluorescence microscope with the rhodamine filter set.

Assessment of yeast viability and metabolic potential. Two methods were used to assess the viability of yeast with VDAC mutations following growth on YPG at 30°C or after a 24-h shift to 37°C. First, the cell density was estimated by measuring the optical density of the culture at 600 nm. The cultures were diluted $1:10^4$, and 100 μl of the diluted culture was spread on a YPD plate. After 2 days at 30°C, the number of colonies was counted and a ratio of the number of colonies to the optical density of the original culture was calculated.

To assess metabolic activity of yeast cells, the cells were stained with the dye FUN-1 (Molecular Probes). This dye is accumulated as orange-red structures in the vacuoles of yeast cells only if they are actively respiring and have intact membranes. The cytoplasm is faintly green in intact cells that are not actively respiring and bright green in dead cells (12). Yeast cells were grown in YPG broth and shifted to 37°C as above. The cells were washed once with 10 mM HEPES (pH 7.2) containing 2% glucose and then resuspended in the same buffer. FUN-1 dye was added to a final concentration of 5 μM and the cells were immobilized with low-melting-point agarose as above. They were viewed after 5 to 10 min by fluorescence microscopy with the fluorescein filter set for the green cytoplasmic fluorescence and the rhodamine filter set for the red vacuolar fluorescence.

Cell fractionation. Total yeast extracts were prepared by the method of Yaffe and Schatz (29). Highly purified yeast mitochondria and subcellular fractions were prepared essentially as described in reference 13. For preparation of outer mitochondrial membranes (OMM), purified mitochondria were pelleted by centrifugation, resuspended in 20 mM HEPES-KOH (pH 7.4)–1 mM phenylmethylsulfonyl fluoride, and incubated for 20 min on ice. The mitochondria were then sonicated, and membranes were collected by centrifugation, resuspended in 20 mM HEPES-KOH (pH 7.4), layered over 1.2 M sucrose–5 mM HEPES-KOH (pH 7.4)–10 mM KCl–1 mM $MgCl_2$–2 mM dithiothreitol, and centrifuged at 40,000 rpm in a T100.3 rotor in a Beckman TL-100 centrifuge. OMM accumulated at the interface between the buffer and sucrose layer. The OMM were then collected, diluted threefold with 20 mM HEPES-KOH (pH 7.4) and collected by centrifugation as described above, and OMM pellets were resuspended in 20 mM HEPES-KOH (pH 7.4).

Reconstitution of VDAC into phospholipid membranes. To generate liposomes containing mitochondrial proteins, 20 mg of egg phosphatidylcholine (Sigma) and 1.75 mg of egg phosphatidylserine (Avanti Biochemicals), both dissolved in chloroform, were dried together under nitrogen. The lipids were then dispersed in 1 ml of 1 mM KCl–1 mM HEPES (pH 7.0) and sonicated to near clarity at 4°C. Mitochondrial membranes prepared as described above were resuspended in 1 mM KCl–1 mM HEPES (pH 7.0) at a protein concentration of 1 mg/ml, 1 ml of this suspension was added to the sonicated lipids, and the mixture was sonicated again. The resulting membranes were then lyophilized. Liposomes were produced by resuspending this material in 1 ml of 20 mM KCl–1 mM EDTA (pH 7.0). A 15-μl volume of this liposome suspension was added to 900 μl of 20 mM KCl–1 mM EDTA (pH 7.0), and the absorbance at 400 nm was monitored as the indicated solutes were added.

YVDAC1 was purified from wild-type yeast mitochondrial membranes or from mitochondria of yeast expressing the *Drosophila* VDAC gene at the *POR1* locus and was introduced into planar phospholipid bilayers as previously described (3).

RESULTS

Library construction and screening. To identify genes which could substitute for *POR1* when overexpressed, a genomic library was prepared from the Δ*por1* yeast strain M22-2 (3) in a multicopy yeast plasmid. Such plasmids contain origins of DNA replication taken from the endogenous yeast plasmid known as 2 μm and therefore are maintained at 20 to 50 copies per cell (5). Consequently, any gene carried on such plasmids is overexpressed in transformed yeast. M22-2 cells were transformed with this library, and eight transformants which could grow on glycerol at 37°C were identified (Fig. 1). Following loss of the library plasmid by extensive growth on nonselective media, each strain was again unable to grow on glycerol-based media at 37°C, indicating that complementation of the growth defect was dependent on the expression of genes contained on the library plasmids. Subsequent restriction analysis indicated that the plasmids recovered from all eight of these clones appeared to contain overlapping fragments of yeast genomic

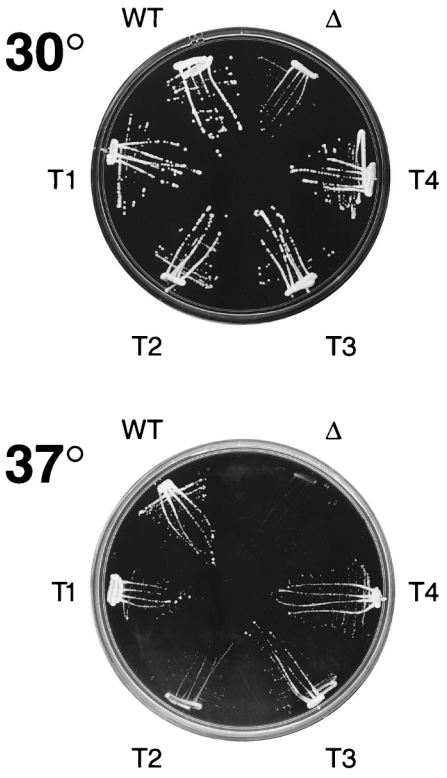

FIG. 1. Multicopy plasmids permit Δ*por1* cells to grow on glycerol at 37°C. Δ*por1* yeast cells with no plasmid (Δ) or containing multicopy plasmids isolated from the Δ*por1* yeast library were streaked on glycerol-containing plates and grown at 30°C (top) or 37°C (bottom). WT, wild-type yeast.

DNA. DNA sequence obtained from the common region of overlap was used to search the National Center for Biotechnology Information database with the BLASTN program. The sequence matched an open reading frame on yeast chromosome IX with homology to *POR1* (cosmid 9150; accession no. z38125). PCR primers based on the sequence at both ends of the open reading frame were used to amplify DNA from all eight complementing plasmids. All eight produced amplification products of the size and restriction map expected based on this common open reading frame. In addition, 2 μm plasmids containing only this open reading frame are sufficient to allow the growth of M22-2 cells at 37°C on glycerol-based media. Thus, each complementing plasmid contained the same gene, which we have named *POR2* and which is entirely responsible for complementation of the growth defects present in Δ*por1* strains. Comparison of the amino acid sequence encoded by the common open reading frame of the *POR2* gene, now designated the YVDAC2 protein, with the sequence of the YVDAC1 protein (Fig. 2) shows that these sequences are 49% identical.

Phenotype of Δ*por* yeast. The *POR2* gene was deleted from wild-type and Δ*por1* yeast cells by homologous recombination (24). Cells with only the *POR2* gene deleted were indistinguishable from wild-type cells in their growth on glucose or glycerol at normal or elevated temperatures (Fig. 3). Cells with both VDAC genes deleted were viable but grew much more slowly than either wild-type or Δ*por1* cells on glucose at 30°C. Double mutants were also able to grow on glycerol-based medium at 30°C, although again more slowly than either wild-type or Δ*por1* cells. As with the Δ*por1* single mutation, they did not grow on glycerol at 37°C (Fig. 3).

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YVDAC1- MSPPVYSDISRNINDLLNKDFYHATPAAFDVQTTTANGIKFSLKAKQPVKDGPL  54
   Con-       ++DISR++N L N+DF+H  P + ++ TTT NG+ F+LKAKQ V +GP+
YVDAC2- MALRFFNDISRDVNGLFNRDFFHTNPLSLNISTTTENGVNFTLKAKQGVTEGPI  54

YVDAC1- STNVEAKLNDKQTGLGLTQGWSNTNNLQTKLEFANLTPGLKNELITSLTPGVAK 108
   Con-  T+VE +  D++ G+ L+Q WSN N L T++EF+ + PG K ++   LTP   K
YVDAC2- QTSVEGRFYDRKEGVSLSQSWSNQNRLNTRIEFSKIAPGWKGDVNAFLTPQSIK 108

YVDAC1- SAVLNTTFTEPFFTARGAFDLCLKSPTFVGDLTMAHEGIVGGAEFGYDISAGSI 162
   Con- +A  N ++ +  F AR + D+ L+   FVG +T+ H G VGG +  YD +AG
YVDAC2- NAKFNLSYAQKSFAARTSIDI-LQPKDFVGSVTLGHRGFVGGTDIAYDTAAGLC 161

YVDAC1- SRYAMALSYFAKDYSLGATLNNEQITTVDFFQNVNAFLQVGAKATMNCKLPNSN 216
   Con- +RYAM++ Y A++YS   + NN Q  T  FFQNVN +LQVG KAT+  K  +SN
YVDAC2- ARYAMSIGYLAREYSFILSTNNRQCATASFFQNVNRYLQVGTKATLQSKT-SSN 214

YVDAC1- VNIEFATRYLPDASSQVKAKVSDSGIVTLAYKQLLRPGVTLGVGSSFDALKLSE 270
   Con- +NIEF TRY+PD+ SQVKAK++DSG+ TL+YK+ L   ++LGVG SF+AL+L+E
YVDAC2- MNIEFVTRYVPDSISQVKAKIADSGLTTLSYKRNLNKDISLGVGMSFNALQLTE 268

YVDAC1- PVHKLGWSLSFDA                                        283
   Con- PVHK GWSLSF
YVDAC2- PVHKFGWSLSFSP                                        281
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FIG. 2. Alignment of *POR1* sequences (above) and *POR2* sequences (below). Sequence identities are indicated by the single-letter amino acid code in the line labeled Con; similar amino acid residues are indicated by +. Dashes in the *POR2* sequence indicate gaps introduced to optimize alignment.

Yeast cells with wild-type VDAC genes or with deletions of one or both *por* genes were grown on glycerol-based medium (YPG) and stained with the mitochondrial vital stain, DASPMI, to examine mitochondrial shape and distribution. In mutant and wild-type cells grown at 30°C and in wild-type and Δ*por2* cells grown at 37°C, mitochondria were observed as bright strings and dots within the cytoplasm (Fig. 4A and B). Mitochondria were observed in the buds of budded cells in these cases, indicating that the Δ*por2* mutation does not prevent the segregation of mitochondria into buds. Mutant Δ*por1* and Δ*por1* Δ*por2* cells were morphologically similar to wild-type cells after growth at 30°C or temperature shifts to 37°C of up to 5 h. However, after 24 h at 37°C, both Δ*por1* and Δ*por1* Δ*por2* cells appeared very heterogeneous, with a few wild-type cells among many large swollen cells containing very large vacuoles (Fig. 4C and D). There were fewer wild-type-appearing cells in double-mutant cultures than in Δ*por1* cultures. In swollen cells, mitochondrial staining was usually observed near the surface of the cell along one side (Fig. 4C and D), but mitochondrial morphology (strings and dots) appeared similar to wild-type morphology. Both wild-type and swollen cells had buds of various sizes, and mitochondria could be observed in buds. Since staining of mitochondria with DASPMI requires the mitochondria to have a transmembrane potential, this result indicates that even abnormal (i.e., swollen) cells contain mitochondria which are still maintaining a membrane potential.

To further investigate the metabolic potential of the mutant cells, the cells were grown on YPG as above and stained with the dye FUN-1. When wild-type, Δ*por1*, or Δ*por2* yeast cells were treated with this dye after growth on YPG at 30°C, >90% of the cells contained red vacuolar structures (Table 1), indicating that they were metabolically active and viable. When cultures of these cells had been shifted to 37°C for 24 h,

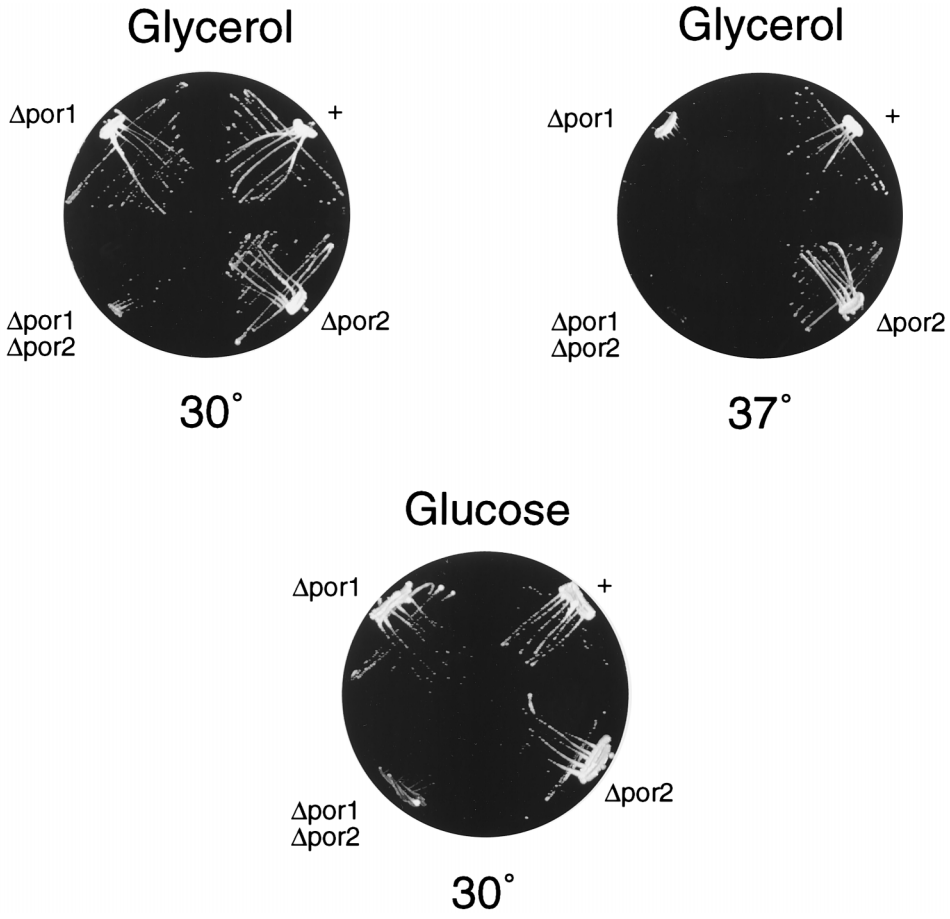

FIG. 3. Growth of Δ*por1* and Δ*por2* yeasts. Wild-type yeast (+) and yeast with the *POR1* gene (Δ*por1*), the *POR2* gene (Δ*por2*), or both genes (Δ*por1* Δ*por2*) deleted were streaked on glycerol- or glucose-based medium and incubated at the indicated temperatures.

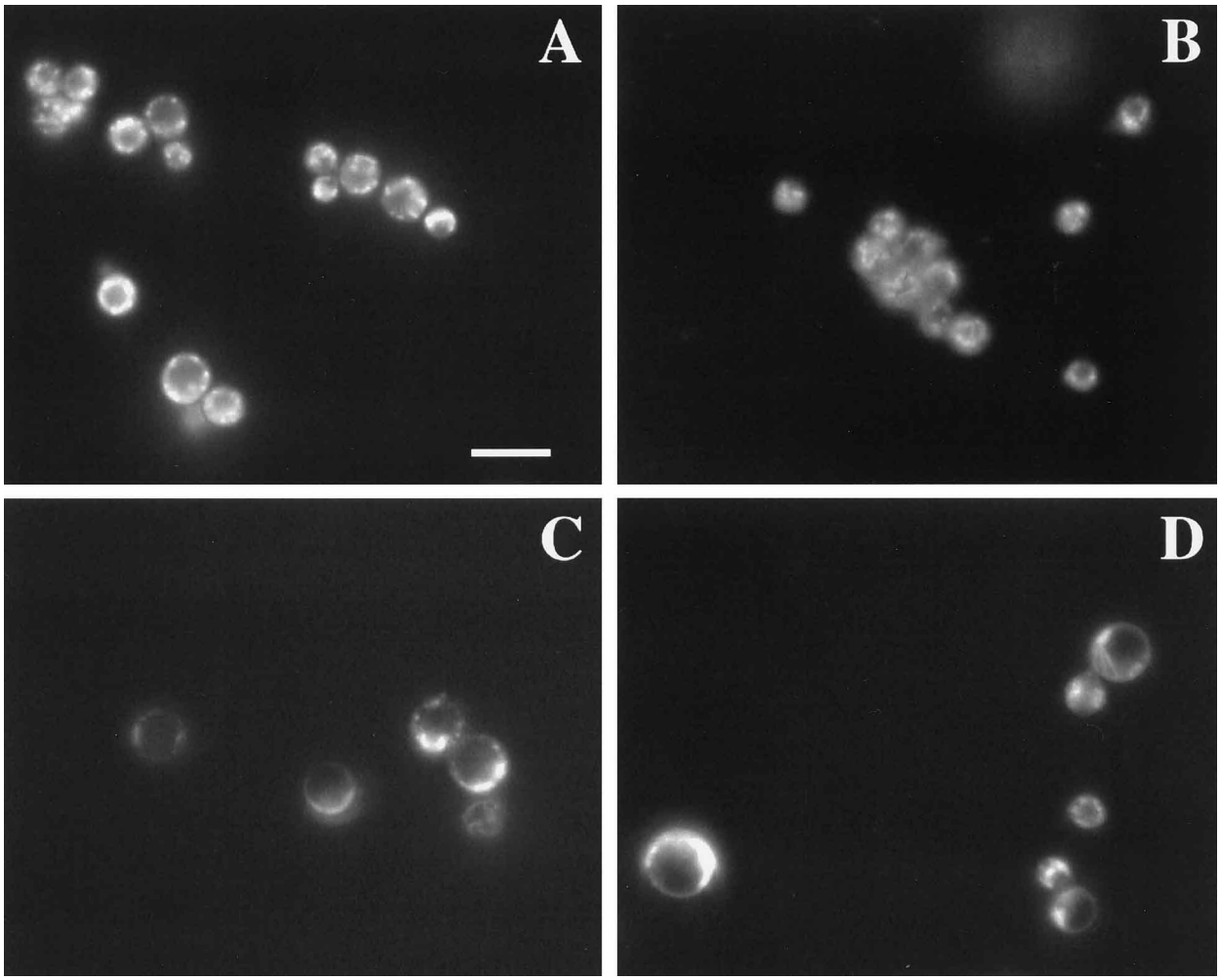

FIG. 4. DASPMI staining of VDAC-deleted yeast cells after growth on glycerol at 37°C. Yeast cells were grown on glycerol-based medium (YPG) to mid-log phase (absorbance at 600 nm, 0.5 to 1.0) at 30°C and then shifted to 37°C for 24 h. (A) Wild type. Mitochondria are visible as bright dots and lines. (B) Δ*por2* yeast. (C) Δ*por1* yeast. Mitochondria are pushed to one side in swollen cells. (D) Δ*por1* Δ*por2* yeast. Mitochondria are pushed to the side but still appear as dots and strings. Note the mitochondria visible in buds. Scale bar, 20 μm. All photographs are at the same magnification.

wild-type and Δ*por2* cells had a similar number of metabolically active cells. This number was slightly reduced in Δ*por1* cells at 37°C. In populations of Δ*por1* Δ*por2* cells, on the other hand, less than half of the cells accumulated dye into vacuolar structures, either at 30 or at 37°C, suggesting that a large number of cells in YPG cultures of the double mutants are either dead or severely impaired in their metabolism.

TABLE 1. Viability of yeast with VDAC deleted after growth on glycerol

Genotype	Growth temp (°C)	FUN-1-positive cells (%)	No. of colonies ml^{-1} $(A_{600})^{-1}$ (10^7)
POR1 POR2	30	93.4	1.8
Δ*por1 POR2*	30	94.2	2.3
POR1 Δ*por2*	30	94.3	2.1
Δ*por1* Δ*por2*	30	31.6	1.8
POR1 POR2	37	96.2	1.6
Δ*por1 POR2*	37	82.0	1.2
POR1 Δ*por2*	37	90.1	1.3
Δ*por1* Δ*por2*	37	48.6	1.4

To distinguish between these alternatives, cells from the same YPG cultures used for FUN-1 staining were diluted and spread on YPD plates to determine the number of viable cells. The colony count was compared to the optical density of the culture to estimate the number of cells capable of forming colonies. As shown in Table 1, the number of viable cells per unit of cell density (as estimated by optical absorbance at 600 nm) was similar for all strains at 30°C. This number was reduced somewhat in cultures grown at 37°C, but even the double mutant was not significantly different from the wild type. In contrast, Δ*por1* Δ*por2* cultures stained with FUN-1 (above) contained substantially fewer metabolically active cells than did cultures of wild-type or single-mutant cells. Thus, the lower percentage of FUN-1-positive cells in Δ*por1* Δ*por2* cultures probably does not reflect an absolute loss of viability on glycerol-based medium but, rather, indicates a metabolically inactive state from which the cells can recover when they are returned to glucose-based medium. This interpretation is also consistent with the ability of a large fraction of Δ*por1* Δ*por2* cells to accumulate DASPMI into mitochondria (see above), suggesting that they are maintaining at least a low level of metabolic activity on glycerol-based medium.

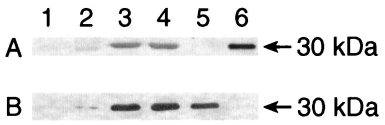

FIG. 5. Immunoblots of subcellular fractions. Wild-type yeast cells were lysed, and soluble cytoplasmic (lane 1), microsomal (lane 2), total mitochondrial (lane 3) and OMM (lane 4) fractions were prepared by differential centrifugation as described in Materials and Methods. The fractions were then separated by SDS-polyacrylamide gel electrophoresis, and the resulting immunoblots were incubated with antisera generated to peptides representing YVDAC2- (A) or YVDAC1 (B)-specific sequences. (A) Lanes 1 to 4 were loaded with 50, 50, 20, and 5 μg of protein, respectively. (B) Lanes 1 to 4 were loaded with approximately 10, 10, 3, and 1 μg of protein, respectively. Lanes 5 and 6 in both panels contain total mitochondrial fractions prepared from Δ*por2* (lane 5; 3 μg of protein) and Δ*por1* (lane 6; 25 μg of protein) cells.

YVDAC2 is located in the mitochondrial outer membrane. To determine the subcellular localization of YVDAC2, wild-type yeast cells were fractionated and highly purified mitochondria and mitochondrial outer membranes were isolated. Western blots of the resulting fractions were probed with antibodies specific for YVDAC1 or YVDAC2 (Fig. 5). The results demonstrate that both YVDAC1 and YVDAC2 are normally found in the mitochondrial and OMM fractions. In addition, as expected from the promoter studies described below, YVDAC1 is normally present in outer membranes at higher levels than YVDAC2, since roughly six to eight times as much of each positive fraction was required to see a YVDAC2-immunoreactive band.

***POR2* complements the Δ*por1* phenotype only when overexpressed.** To determine whether a single extra copy of the *POR2* gene could restore growth on glycerol at 37°C to Δ*por1* cells, the *POR2* gene was inserted into a centromere-based yeast vector that is maintained at one or two copies per cell. When this plasmid was introduced into Δ*por1* (Fig. 6A and B) or Δ*por1* Δ*por2* (Fig. 6C) cells, transformants failed to grow on glycerol at 37°C (Table 2). However, when the promoter region of *POR1* was placed upstream of the *POR2* coding sequence in a centromere-based plasmid or when such constructs were reintroduced as a single copy into the genome at the *POR1* locus, Δ*por1* (Fig. 6D) and Δ*por1* Δ*por2* (Fig. 6C) cells regained the ability to grow on glycerol at 37°C. In addition, Δ*por1* cells transformed with multicopy plasmids containing the promoter region of *POR2* upstream of the *POR1* coding sequence grew as well as wild-type cells at 37°C on glycerol (Table 2). These results suggest that the *POR1* promoter mediates higher levels of expression than the *POR2* promoter and that rescue of Δ*por1* growth on glycerol-based medium at the restrictive temperature by the *POR2* gene requires overexpression of *POR2*, either by the presence of multiple copies of the *POR2* gene or by expression of *POR2* to levels similar to *POR1* by use of the *POR1* promoter. This idea was confirmed by comparing levels of *POR1* expression mediated either by its own promoter or by the *POR2* promoter on centromere-based plasmids and expression from the *POR2* promoter on a multicopy plasmid. Western blots of total cell extracts prepared from Δ*por1* cells containing these constructs and probed with a YVDAC1-specific antibody demonstrate that the *POR1* promoter mediates much higher levels of expression than the *POR2* promoter from centromere-based plasmids while the *POR2* promoter mediates high levels of *POR1* expression only when included in a multicopy plasmid (Fig. 7, lanes 1 to 4). Similarly, when a construct containing the YVDAC2 coding region under the control of the *POR1* promoter was integrated into the genome, the expression of YVDAC2 was substantially higher than from the chromosomal copy of the *POR2* gene with its own promoter (lanes 5 and 6). This higher expression level was observed whether the cells were grown on glucose or glycerol-based medium (lanes 5 to 8).

Yeast containing a single-copy plasmid in which the *POR2* promoter drives the expression of *POR1* grew somewhat better than Δ*por1* cells but more slowly than wild-type cells or cells in which the Δ*por1* growth defect is rescued by the expression of adequate levels of YVDAC1 or YVDAC2 (Table 2; Fig. 6D). Thus, the low level of YVDAC1 expression from the *POR2* promoter compensates better for lack of YVDAC1 than does the low level of YVDAC2 produced by the same promoter.

YVDAC2 does not appear to form channels. VDAC molecules with widely different primary amino acid sequences isolated from an array of organisms (yeasts, mammals, and plants) have been observed to form voltage-dependent, anion-selective channels when purified from mitochondria or OMM and incorporated into planar phospholipid membranes. However, despite an intense effort involving a variety of different expression approaches in both yeasts and bacteria and a number of purification techniques, it has not been possible to generate channels from YVDAC2 proteins in planar phospholipid bilayers. Therefore, an alternate approach was used to examine the ability of YVDAC2 proteins to form channels. Mitochondrial membranes from yeast strains containing the *POR1* and *POR2* genes in various combinations were each incorporated into liposomes, and the resulting vesicles were examined for their ability to swell and shrink in the presence of nonelectrolytes of various sizes. The optical absorbance of a liposome suspension is correlated with the volume of the liposomes such that the absorbance of such suspensions increases or decreases in response to liposome shrinkage or swelling, respectively. Thus, the permeability of the liposomes to nonelectrolytes with various Stokes-Einstein radii (Table 3) can be assessed by monitoring the optical absorbance of the liposome suspension following addition of a solution of the nonelectrolyte. If the nonelectrolyte is completely impermeable, the liposomes shrink (absorbance increase) as water is drawn out until the water activity is balanced. If a given solute is permeable, the liposomes first shrink (absorbance increases) and then reswell (absorbance decreases) as water accompanies the solute back into the liposomes.

In Fig. 8A, liposomes containing proteins from the mitochondrial membranes of wild-type yeast cells increase in absorbance following the addition of polyethylene glycol (PEG) of three different sizes. This initial increase, corresponding to liposome shrinkage from water loss, is followed by a gradual decrease in absorbance following addition of either PEG 1500 or PEG 3400, indicating that these solutes enter the liposomes and cause reswelling. In the case of PEG 6800, the initial shrinkage due to water loss is seen but no reswelling follows, indicating that the liposomes are impermeable to this solute. Thus, the permeability pathways reconstituted in these liposomes have a molecular size cutoff radius of about 1.9 nm, consistent with previous results obtained for mitochondrial membranes by this and similar techniques and consistent with the accepted properties of VDAC (6, 20). Figure 8B and C shows the responses of liposomes containing mitochondrial membranes from Δ*por1* and Δ*por1* Δ*por2* yeast cells, respectively. Both are permeable to stachyose and raffinose but not to γ-cyclodextrin or PEG 1500. Thus, their permeability has a cutoff radius of around 0.7 nm. Similar results were observed for liposomes containing mitochondrial protein prepared from Δ*por1* yeast cells overexpressing YVDAC2 (Fig. 8D).

Channel formation is separable from the ability to complement the growth defect. The inability of YVDAC2 to form channels in either bilayers or liposomes raises the possibil-

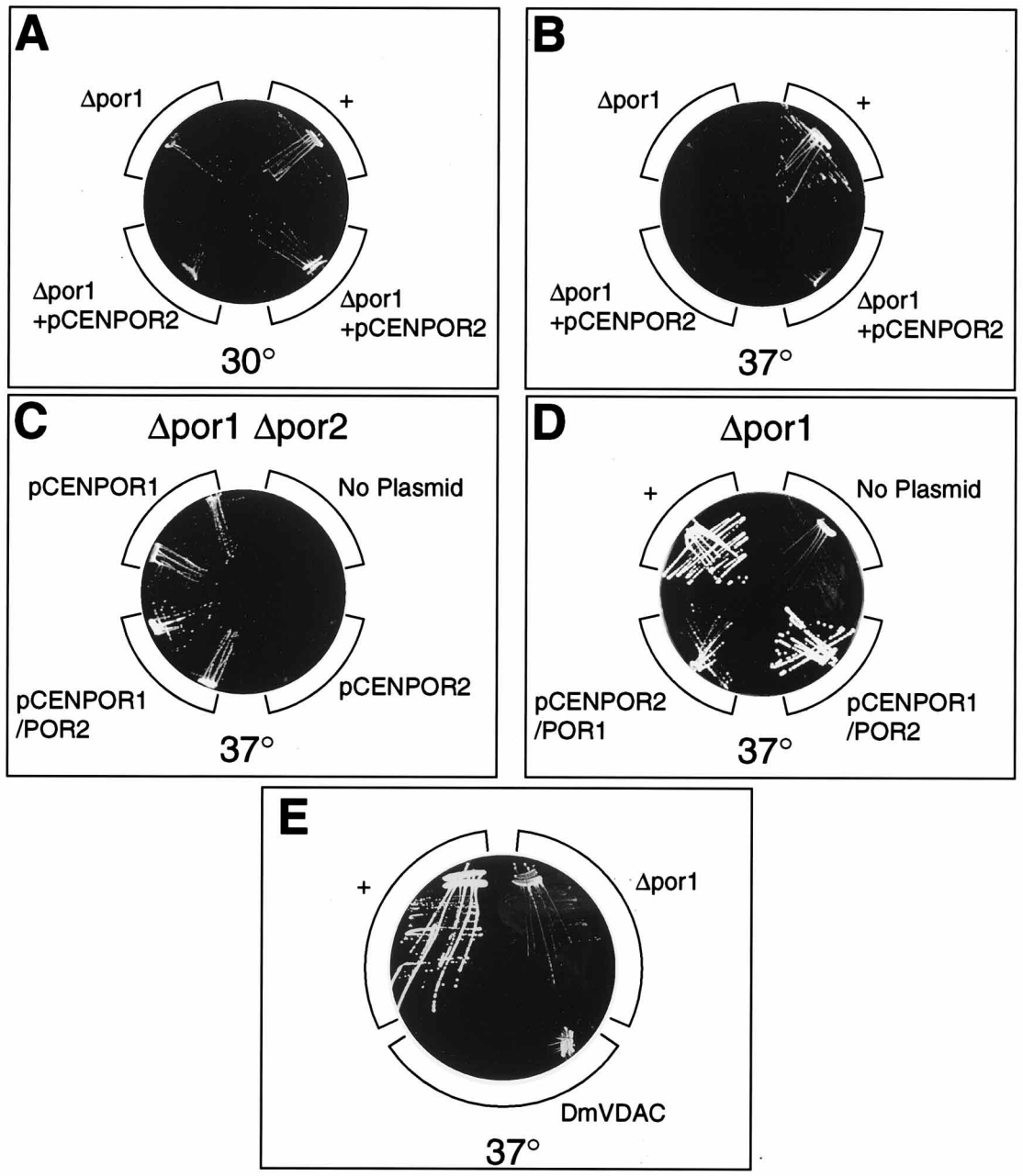

FIG. 6. Complementation of the Δ*por1* phenotype by single-copy plasmids. (A and B) Δ*por1* yeast cells were transformed with a centromere plasmid containing the POR2 gene, and streaked on glycerol-containing plates, and grown at 30°C (A) or 37°C (B). Wild-type cells are indicated by +, and untransformed Δ*por1* cells are indicated by Δ*por1*. (C) Yeast cells lacking both VDAC genes (Δ*por1* Δ*por2*) were transformed with a centromere plasmid containing the *POR1* gene (pCENPOR1), a centromere plasmid containing the *POR2* gene (pCENPOR2), or a centromere plasmid containing the *POR2* coding region under the control of the *POR1* promoter (pCENPOR1/POR2). Transformants were streaked on glycerol-containing plates and grown at 37°C. (D) Δ*por1* cells were transformed with centromere plasmids containing either the *POR2* coding region under the control of the *POR1* promoter (pCENPOR1/POR2) or the *POR1* coding region under the control of the *POR2* promoter (pCEN POR2/POR1). Transformants were streaked on glycerol-containing plates and grown at 37°C. No Plasmid indicates the untransformed Δ*por1* strain; + indicates the wild-type strain. (E) A construct for expressing the *Drosophila* VDAC from the *POR1* promoter was integrated into yeast M22-2. The resulting strain (DmVDAC) was streaked on glycerol plates and grown at 37°C with wild-type and M22-2 controls.

ity that the ability to complement the temperature-sensitive growth of Δ*por* mutants on nonfermentable carbon results from some function other than channel formation. To address this question, we attempted to identify VDAC molecules which were capable of forming channels but which were unable to complement growth defects present in Δ*por* strains. A clear example is provided by VDAC from *D. melanogaster* (DmVDAC). DmVDAC is roughly 30% identical to YVDAC1. When the DmVDAC gene was expressed in Δ*por1* cells from the POR1 promoter, cells failed to grow at 37°C on nonfermentable carbon sources (Fig. 6E; Table 2) (25). In fact, Δ*por1* yeast expressing DmVDAC grow more poorly on glycerol, even at 30°C, than did Δ*por1* yeast (data not shown). Nevertheless, DmVDAC is synthesized and localized to the

TABLE 2. Growth of yeast with VDAC deletions and complementation by VDAC plasmids

Strain genotype	Plasmid			Growth on YPG at 37°C[a]
	Promoter	Coding region	No. of copies	
POR1 POR2				+
POR1 Δ*por2*				+
Δ*por1 POR2*				−
Δ*por1* Δ*por2*				−
PPOR1 POR2 POR2				+
PPOR1 DmVDAC POR2				−
Δ*por1 POR2*	*POR1*	*POR1*	1–2	+
Δ*por1 POR2*	*POR1*	*POR1*	Multiple	+
Δ*por1 POR2*	*POR1*	*POR2*	1–2	+
Δ*por1 POR2*	*POR1*	*POR2*	Multiple	+
Δ*por1 POR2*	*POR2*	*POR1*	1–2	±
Δ*por1 POR2*	*POR2*	*POR1*	Multiple	+
Δ*por1 POR2*	*POR2*	*POR2*	1–2	−
Δ*por1 POR2*	*POR2*	*POR2*	Multiple	+
Δ*por1* Δ*por2*	*POR1*	*POR1*	1–2	+
Δ*por1* Δ*por2*	*POR1*	*POR2*	1–2	+
Δ*por1* Δ*por2*	*POR2*	*POR1*	1–2	±
Δ*por1* Δ*por2*	*POR2*	*POR2*	1–2	−

[a] +, growth similar to wild-type growth; −, growth similar to Δ*por1* strain growth; ±, intermediate growth.

mitochondria in these yeast cells (Fig. 7, lane 9). When isolated and purified from mitochondria, DmVDAC forms channels in planar membranes which have characteristics identical to YVDAC1 channels (Fig. 9) or channels formed by other VDAC molecules (6, 20). Likewise, when mitochondrial membranes from Δ*por1* yeast expressing DmVDAC were introduced into liposomes, they exhibited a permeability to large nonelectrolytes similar to that of wild-type yeast mitochondria (Fig. 8E). Thus, the single-channel conductance, voltage dependence, and ability to allow the passage of nonelectrolytes of DmVDAC are essentially identical to YVDAC1, yet expression of this protein does not complement the growth defects present in Δ*por* strains. In contrast, YVDAC2 apparently does not form channels but does complement the Δ*por* growth defect when expressed at similar levels. Thus, complementation of Δ*por* growth defects on nonfermentable carbon sources appears to require a set of functions provided by VDAC molecules that can be distinguished from the ability to form channels.

FIG. 7. Immunoblots of YVDAC1 and YVDAC2 expressed from different promoters. In lanes 1 to 8, whole-cell extracts (29) were prepared from 1 absorbance unit at 600 nm each of cultures of cells containing various YVDAC expression constructs, and an equal fraction of each extract was fractionated on SDS-polyacrylamide gels. The blots were immunostained using antibody to YVDAC 1 (lanes 1 to 4) or to YVDAC2 (lanes 5 to 8). Lanes 1 to 4 contain Δ*por1* cells with YVDAC plasmids grown on glucose-based media: lane 1, vector alone; lane 2, single-copy plasmid containing the *POR1* coding region under control of the *POR2* promoter; lane 3, single-copy plasmid containing the *POR1* gene with its own promoter; lane 4, multicopy plasmid containing the *POR1* coding region with the *POR2* promoter. Lanes 5 to 8 contain Δ*por1* cells (lanes 5 and 7) or Δ*por1* cells with a single copy of the *POR2* coding region under the control of the *POR1* promoter integrated into the chromosome (lanes 6 and 8) grown on glucose- (lanes 5 and 6) or glycerol (lanes 7 and 8)-based medium. Due to the low levels of expression driven by the *POR2* promoter, no signal is visible at this level of loading from cells containing *POR1* coding sequences driven by the *POR2* promoter (lane 2). Lane 9, washed mitochondria were prepared from Δ*por1* cells expressing the *Drosophila* VDAC gene from the POR1 promoter, dissolved in SDS sample buffer, and fractionated on an SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose and immunostained with antibody to *Drosophila* VDAC.

TABLE 3. Comparison of Stokes-Einstein radius of nonelectrolytes

Solute	Mol wt	Stokes-Einstein radius
Raffinose	504	0.57
Stachyose	667	0.66
γ-Cyclodextrin	1,297	0.84
PEG 1500	1,500	1.2
PEG 3400	3,400	1.9
PEG 6800	6,800	2.9

DISCUSSION

All metazoan organisms examined to date contain a family of genes which encode a variety of different yet highly related isoforms of VDAC. For example, in humans, at least four different genes encoding VDAC isoforms have been identified, and the two that have been examined in greatest detail, HVDAC1 and HVDAC2, were found to be closely related (~70% identical) (4). Multiple isoforms of VDAC have also been identified in other mammals (26) and plants (9, 15). However, a number of previous studies have indicated that single-celled organisms such as the yeast *S. cerevisiae* contain only one VDAC gene. These results predicted that Δ*por1* cells would have respiratory deficiencies, since VDAC is thought to provide the primary pathway for the movement of metabolites across the OMM. However, cells lacking this gene were found to be competent for respiration as assessed by their ability to grow on nonfermentable carbon sources like glycerol, although their growth on these carbon sources is temperature sensitive. This suggested that there are molecules which can compensate for the absence of the VDAC protein encoded by the *POR1* gene at the permissive temperature. The genetic studies described here have resulted in the identification of a gene which we have designated *POR2* and which encodes a VDAC-like molecule, YVDAC2. The protein encoded by *POR2* is more distantly related (49% identical) to the original yeast VDAC gene, *POR1*, than are related isoforms in metazoans (i.e., HVDAC1 and HVDAC2). This relatively low level of homology is likely to explain why the *POR2* gene was not detected by Southern blot analysis with *POR1* probes or by cross-reaction with antibodies directed against the YVDAC1 protein. In spite of the divergence in sequence between the YVDAC1 and YVDAC2 proteins, these proteins are more similar to each other than either is to any other identified VDAC gene. In addition, given the number of genome equivalents screened (>50) and the fact that all eight complementing clones identified contained only the YVDAC2 gene in common, it is unlikely that additional genes exist in the yeast genome which alone can compensate for the growth defect, on glycerol-based medium, of Δ*por1* cells when they are overexpressed. Thus, since YVDAC2 alone permits the growth of Δ*por1* cells on nonfermentable carbon sources at the restrictive temperature, it is reasonable to conclude that YVDAC2 must be the only gene able to replace functions that are normally provided by YVDAC1 under these conditions and also that all eukaryotic organisms probably express a family of VDAC-like proteins.

The existence of a family of genes encoding VDAC molecules in multicellular organisms has been interpreted to suggest that VDAC may be present in cell compartments other than the mitochondria (see, e.g., reference 22). Thus, it was possible that the YVDAC2 protein was normally located in a cell membrane other than the outer mitochondrial membrane

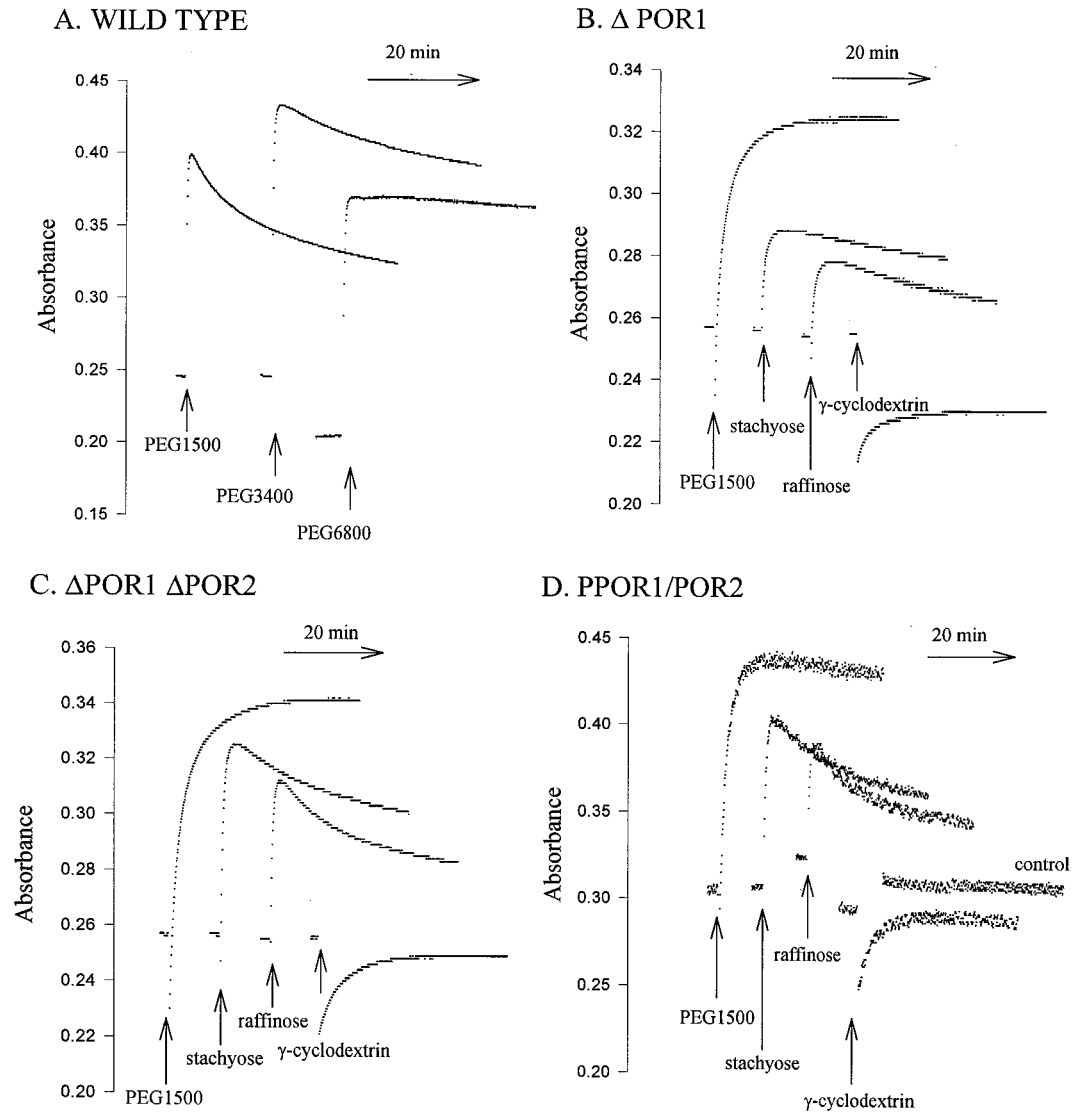

FIG. 8. Response of liposomes containing yeast mitochondrial membrane proteins to osmotic pressure gradients induced with nonelectrolytes with different molecular weights. Mitochondrial membranes were isolated from wild-type (A), Δ*por1* (B), or Δ*por1* Δ*por2* (C) cells or cells overexpressing *POR2* by virtue of the insertion at the *POR1* locus of a construct in which expression of the *POR2* coding sequence is directed by the *POR1* promoter (*PPor1*/*Por2*) (D) cells expressing *Drosophila* VDAC from the *POR1* promoter at the *POR1* locus (E). After the absorption of the 0.92-ml liposome suspension had stabilized, one of the following solutions of nonelectrolytes was added at the time point indicated: 35 μl of 1.0 M raffinose; 35 μl of 0.73 M stachyose; 220 μl of 20 mM γ-cyclodextrin; 67 μl of 100 mM PEG 1500; 67 μl of 100 mM PEG 3400; or 67 μl of 50 mM PEG 6800. In panel D, all the volumes were trebled and the cuvette was stirred continuously to avoid the propensity of the liposomes to settle. This agitation caused the additional noise in these records.

and that overexpression resulted in redistribution of the protein to the outer membrane, where it could compensate for the Δ*por1* growth defect. Immunoblots of subcellular fractions prepared from wild-type cells probed with isoform-specific antibodies indicate that both YVDAC1 and YVDAC2 are normally located in the OMM. However, these results also indicate that most of the VDAC in the OMM of wild-type cells is the product of the *POR1* gene. Consistent with the major role of YVDAC1, deletion of the *POR2* gene alone appears to

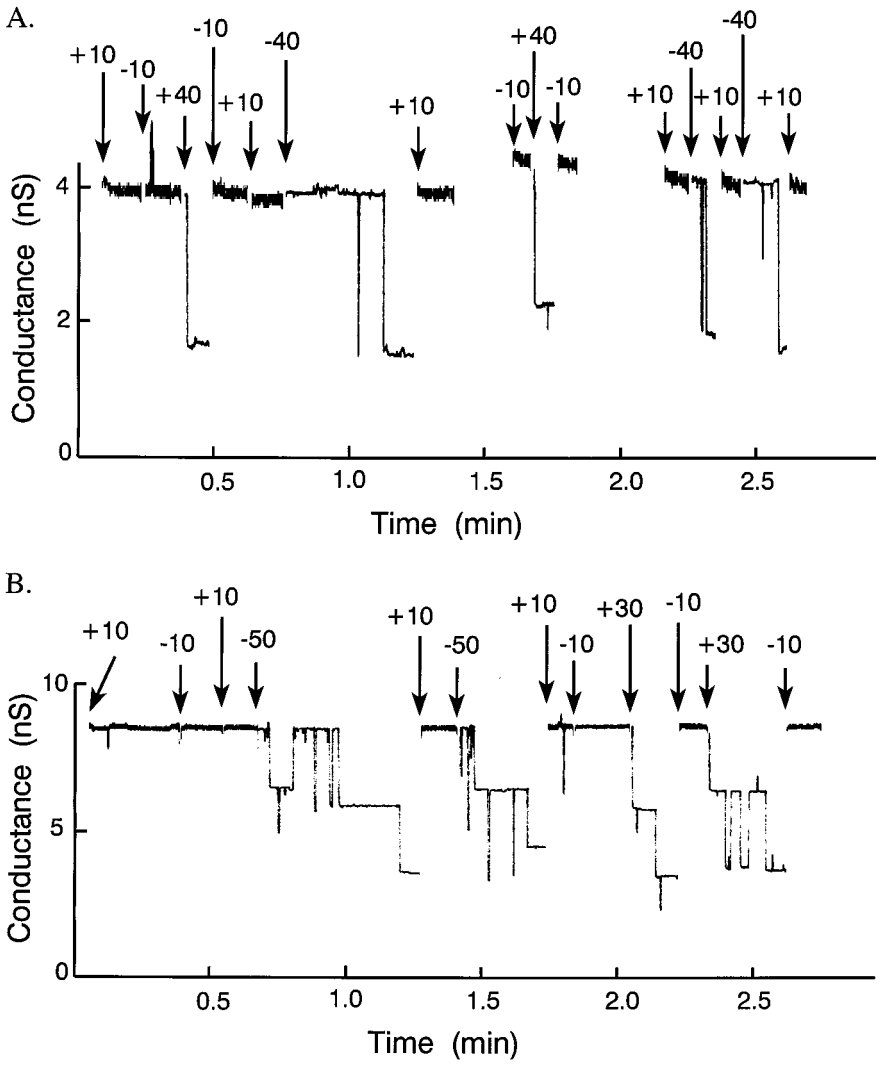

FIG. 9. *Drosophila* VDAC channels resemble YVDAC1 channels. Single-channel traces of YVDAC1 (A, one channel) or *Drosophila* VDAC (B, two channels) are shown. VDAC was purified from yeast mitochondria and inserted into synthetic lipid bilayers. Conductance is displayed as a function of time. At low potentials (+10 or −10 mV), the channels remain in the high-conductance (open) state. At higher potentials (+30 to 40 mV, −40 to 50 mV), the channels close to a lower-conductance state or alternate between the open and closed states.

be without consequence for growth at either 30 or 37°C on either glucose or glycerol-based medium. The difference in the level of expression appears to be due in part to the ability of the *POR1* promoter to drive higher levels of expression than the *POR2* promoter.

Since VDAC molecules are thought to function as pathways for the permeation of charged solutes across the OMM, suppression by YVDAC2 of growth defects present in Δ*por1* cells would predict that YVDAC2 is also able to mediate the movement of such compounds across the OMM. In this case, deletion of both *POR1* and *POR2* genes should result in the complete absence of any pathway for the movement of metabolites across this membrane and thus the complete inability to grow on nonfermentable carbon sources. However, Δ*por1* Δ*por2* cells are able to grow on glycerol at 30°C. Growth is clearly impaired under these conditions; mutant cells grow slowly, and FUN-1 staining indicates that more than half of the cells are metabolically inactive. However, Δ*por1* Δ*por2* cells do not die on glycerol-based media, since they are capable of forming colonies when shifted to glucose. Further, the ability of mitochondria in these cells to take up DASPMI is consistent with the idea that the mitochondria are able to produce a membrane potential but not to convert the energy of the potential into ATP, a situation that would be expected if the movement of metabolites through the outer membrane was reduced. Thus, these results indicate that the growth characteristics of Δ*por* cells do not reflect an absolute loss of viability on glycerol-based medium but, rather, that cells enter a metabolically inactive state from which they can recover when returned to glucose-based medium.

The fact that Δ*por1* Δ*por2* cells can grow at a low rate on glycerol indicates that other pathways for the movement of these molecules into and out of mitochondria must exist. However, the slower growth of Δ*por1* Δ*por2* cells would also suggest that these pathways are not as efficient as those provided by either YVDAC1 or YVDAC2. Thus, these results lead to the conclusion that there must be additional functional redundancy that allows mitochondria to continue oxidative phosphorylation even in the absence of both VDAC proteins, calling into question the generally held unique role of these

proteins in mitochondrial function. Examination of the complete sequence of the yeast genome suggests that the molecule(s) which compensates for the lack of both VDAC genes is not closely related to either of these genes. However, previous results have demonstrated that even distantly related (20% homology) human VDAC genes can functionally compensate for the absence of the yeast *POR1* gene (4). In addition, unlike YVDAC2, yeast proteins forming these alternate permeability pathways must be unable to suppress the growth defects of Δ*por1* cells when they are overexpressed, since they were not identified in our multicopy suppressor screens.

Complementation of the growth defect of Δ*por1* cells by YVDAC2 requires overexpression of this protein, either by inclusion of the *POR2* gene on a multicopy plasmid or by expression of the YVDAC2 protein to levels similar to that of the YVDAC1 protein by use of the *POR1* promoter. This indicates that there must be an overlap in function between the two genes. Further, this observation and the fact that Δ*por1* and Δ*por1* Δ*por2* yeast cells are unable to grow on glycerol at 37°C indicate that growth of yeast cells under these conditions requires a critical level of either VDAC protein. However, the introduction of single-copy plasmids in which YVDAC1 expression is mediated by the *POR2* promoter allows Δ*por1* cells to grow better on glycerol at the restrictive temperature than does an equally low level of YVDAC2 produced by the same promoter. This suggests that YVDAC1 and YVDAC2 may have different although overlapping functions.

Despite repeated attempts, we have been unable to observe YVDAC2 channels in planar phospholipid membranes by using a wide variety of protocols that have been successfully used in the analysis of VDAC proteins from organisms as divergent as yeasts, plants, and humans. Although there are many possible explanations for this failure, one possibility is that YVDAC2 does not normally form channels and that it can provide a YVDAC1 function that does not involve channel formation. To test this idea, mitochondrial membranes from yeast strains expressing YVDAC1 and YVDAC2 separately or in combination were incorporated into liposomes and the resulting vesicles were examined for their ability to swell and shrink in the presence of nonelectrolytes of various sizes. The results indicate that yeast mitochondrial membrane permeability pathways that allow the passage of compounds with a radius larger than 0.7 nm can be attributed to YVDAC1 proteins only. Further, the properties of membranes prepared from Δ*por1* and Δ*por1* Δ*por2* cells indicate that the residual permeability of these membranes is due to a molecule other than YVDAC2, most probably one of the other channels that have been identified in mitochondrial membranes (19). Thus, YVDAC2 does not appear to be capable of forming a pathway for the movement of even small solutes. Alternatively, it is still formally possible that YVDAC2 can form channels in the outer membrane but that the channels require an additional factor to function, or that the majority of the overexpressed YVDAC2 protein is folded incorrectly although inserted into the outer membrane. In either of these cases, the level of functional YVDAC2 channels would be much lower than would be expected on the basis of Western blots and thus would be undetectable by the methods we have used to assess channel-forming ability. This low level of functional channels might be sufficient to allow cells to grow on glycerol, although slowly.

However, our results with expression of the *Drosophila* VDAC protein in yeast suggest that the ability to complement the temperature-sensitive growth defect of Δ*por1* yeast is separate from the ability to form channels. The *Drosophila* VDAC protein can be expressed in Δ*por1* yeast cells and is localized to the mitochondrial membranes. When purified from these membranes and introduced into lipid bilayers, it forms channels indistinguishable from those of YVDAC1, yet this protein will not complement the growth defect of Δ*por1* yeast on glycerol at 37°C. Similar studies have demonstrated that VDAC proteins purified from the mitochondria of a number of plants are able to generate characteristic VDAC channels but unable to complement Δ*por1* growth defects when expressed in these cells (15). This difference is not likely to be due to the absolute difference in amino acid sequence identity between the YVDACs and DmVDAC, since the expression of VDACs with similar (*Neurospora crassa* [2a]) or lower (human [4]) levels of identity allow the growth of Δ*por1* cells at 37°C on glycerol-based media. Thus, the inability of DmVDAC to complement growth defects present in Δ*por1* cells probably represents the absence of specific sequences or structural elements in DmVDAC that are present in YVDACs and other VDAC molecules.

Based on these observations, channel-forming activity does not seem to be either necessary or sufficient to restore growth on nonfermentable carbon sources, leading to the conclusion that VDAC mediates additional cellular functions that do not depend on the ability to form channels which provide a pathway for the movement of charged molecules across the outer membrane. The failure of Δ*por1* cells to grow on glycerol at the restrictive temperature may be caused by the loss of these "nonchannel" functions normally provided by YVDAC1, and growth under these conditions can be restored by adequate levels of either YVDAC1 or YVDAC2. In this case, YVDAC1 would be able to form channels as well as to provide the additional function, while YVDAC2 could perform only the second function.

A number of previous studies have suggested cellular roles for VDAC that do not require channel function. For example, it is known that in mammalian cells, VDAC is the binding site for cytoplasmic kinases such as hexokinase and glycerol kinase, which bind reversibly to the outer surface of the OMM, which may allow them preferential access to mitochondrially produced ATP (1). VDAC may also be involved in the association of mitochondria with the cytoskeleton (18), although our results indicate that VDAC is not necessary for mitochondrial segregation into the bud. YVDAC2 may have diverged evolutionarily from YVDAC1 such that it has lost the ability to form channels but still functions, for example, as a binding site for molecules which must associate with the mitochondrion. In addition, this view would imply that the ability of distantly related VDAC molecules (e.g., HVDAC1) to rescue the growth defect of Δ*por1* cells depends not on their channel-forming ability, although some molecules that rescue the growth defect have been shown to form channels, but on their ability to provide functions which do not require channel activity. This may explain why multicellular organisms contain multiple VDAC genes. Thus, not all VDAC molecules identified by molecular techniques are necessarily also channel-forming molecules. Studies which should provide insights into potential role of this family of molecules in other cellular processes in addition to the formation of permeability pathways for the movement of metabolites across the OMM are under way.

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