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Functional Characterization of a Second Porin Isoform in *Drosophila melanogaster*

DMPORIN2 FORMS VOLTAGE-INDEPENDENT CATION-SELECTIVE PORES*

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Mitochondrial porins or voltage-dependent anion-selective channels are channel-forming proteins mainly found in the mitochondrial outer membrane. Genome sequencing of the fruit fly *Drosophila melanogaster* revealed the presence of three additional porin-like genes. No functional information was available for the different gene products. In this work we have studied the function of the gene product closest to the known *Porin* gene (*CG17137* coding for DmPorin2). Its coding sequence was expressed in *Escherichia coli*. The recombinant DmPorin2 protein is able to form channels similar to those formed by DmPorin1 reconstituted in artificial membranes. Furthermore, DmPorin2 is clearly voltage-independent and cation-selective, whereas its counterpart isoform 1 is voltage-dependent and anion-selective. Sequence comparison of the two porin isoforms indicates the exchange of four lysines in DmPorin1 for four glutamic acids in DmPorin2. We have mutated two of them (Glu-66 and Glu-163) to lysines to investigate their role in the functional features of the pore. The mutants E163K and E66K/E163K are endowed with an almost full inversion of the ion selectivity. Both single mutations partially restore the voltage dependence of the pore. We found that an additional effect with the double mutant E66K/E163K was the restoration of voltage dependence. Protein structure predictions highlight a 16 β-strand pattern, typical for porins. In a three-dimensional model of DmPorin2, Glu-66 and Glu-163 are close to the rim of the channel, on two opposite sides. DmPorin2 is expressed in all the fly tissues and in all the developmental stages tested. Our main conclusions are as follows. 1) The *CG17137* gene may express a porin with a functional role in *D. melanogaster*. 2) We have identified two amino acids of major relevance for the voltage dependence of the porin pore.

Mitochondrial porins or voltage-dependent anion-selective channels (VDACs)[1] are pore-forming proteins mainly found in the mitochondrial outer membrane, where they are able to form large hydrophilic channels (1–3). Although many features of this protein family have been known for more than 20 years, the atomic structure is still lacking, and only models have been computed according to secondary structure predictions (4–7). All models indicate that the transmembrane segments typical for these channels are formed by antiparallel, amphipathic β-strands, similar to the situation in bacterial porins (8). Porin or VDAC is the major pore-forming protein of the mitochondrial outer membrane and has a unique role in energetic metabolism since all metabolites, including ADP and ATP, have to pass through the porin channels (9, 10). It is the receptor where kinases bind to the mitochondrial surface, which is also important for the metabolic energy channeling in the cell (11). The role of mitochondrial porin isoform 1 or VDAC1 in apoptosis was also thoroughly investigated in the last few years (12–16). Its participation in the permeability transition pore has been reported (17–19). A recent report (20) focused on the VDAC2 isoform as an important player in the cell death pathway.

In the last years it has been demonstrated that the chromosomes of eukaryotic cells contain several genes coding for porins. In *Saccharomyces cerevisiae* the deletion of the *porin* gene is not lethal for the cells unless they were forced to live under strictly mitochondrially driven, aerobic conditions (21). This observation led to the discovery of a second gene, coding for a porin analogue and termed VDAC2 by sequence homology (22). Although the yeast VDAC2 is unable to form channels *in vitro*, its overexpression complements the inability to grow on nonfermentable carbon sources, the cellular defect induced by the VDAC1 deletion (23).

The genomes of humans, rats, and mice contain three different porin genes coding for distinctly expressed isoforms (23–25). Human and mouse VDAC1 and VDAC2 isoforms show channel forming activity *in vitro* and can complement the yeast VDAC1 deficiency, whereas VDAC3 can only partially complement this defect (25). An interesting picture of the porin genes in a higher eukaryotic organism has been revealed in the fruit fly *Drosophila melanogaster*. The *porin* gene, which codes for DmPorin1, was cloned, sequenced, and mapped at 32B3-4 on the second chromosome. It has an identity of about 60% compared with those of the porin isoforms in mammals (26, 27).

The organization of the region adjacent to the *porin* locus

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[1] The abbreviations used are: VDAC, voltage-dependent anion-selective channel; EST, expressed sequence tag; for, forward; rev, reverse; nS, nanosiemens; Dm, *D. melanogaster*.

TABLE I
Primers used for amplification of DmPorins and mutants

The mutagenized nucleotides are in boldface type. The double mutant E66K/E163K was obtained by applying mutagenesis to the single mutant E66K with the primers E163Kfor and E163Krev.

Primer	Sequence
DmPorin1for	CGC GGA TCC ATG CTC CTC CAT CAT ACAG
DmPorin1rev	CGC AAG CTT TTA TTA GGC CTC CAG CTC CAG
DmPorin2for	TAG GGA TCC ATG GCC GCC AAA ACA CCG ACA
DmPorin2rev	TAG AAG CTT TTA TAT TGC CAT TAT GAA GAA
E66Kfor	AAG TAC AAG ATC **A**AG GAC CAA GGC CTG
E66Krev	CAG GCC TTG GTC CT**T** GAT CTT GTA CTT
E163Kfor	TGG CAA CAC CA**A** ACT CAA GGG
E163Krev	CCC TTG AGT **T**TG GTG TTG CCA
Rp49for	ATA GGA CTT ATG ACC ATC CGC CCA GCA TAC
Rp49rev	CGC AAG CTT TTA CCT CGT TCT TCT TGA GAC

was elucidated after completion of the fruit fly genome. Most surprising, it revealed the close proximity of three additional genes with significant homology to the *porin* gene called *CG17137*, *CG17139*, and *CG17140* (28). These three genes were predicted *in silico* by alignment of the genomic sequences with the EST sequences in the Berkeley *Drosophila* Genome Project collection. They are on the same strand as the *porin* gene, and they have a similar organization with two introns interrupting the coding sequence (28). The 5′-untranslated region is present only in the *porin* gene. The nucleotide distances separating the four *porin*-like genes are extremely reduced. EST sequences have been reported for *CG17137* and for *CG17139* and *CG17140*, albeit considerably less frequent than for the *porin* gene. In a most recent update of the Flybase Data Bank (FLYBASE, FBgn0051722; LocusID, 34497), the putative genes *CG17139* and *CG17140* have been considered as a single transcription unit termed *CG31722*, since no 3′-EST is available for the gene *CG17139*.

The expression of *CG17137* and *CG31722* is established at the transcriptional level, since the corresponding ESTs have been identified. Alignment of the deduced protein sequences shows sequence conservation in pairs. The protein sequence deduced from *CG17137* is 42% identical to *D. melanogaster* porin isoform 1, but it is only 26% identical to the deduced amino acid sequence of *CG17140* or *CG31722* (28).

In this paper we have cloned the coding sequence corresponding to *CG17137* and expressed it in *Escherichia coli*. The expressed protein was purified and refolded. It showed pore forming activity with features resembling but not overlapping those of the isoform 1. In addition, the functional characterization revealed new features that could be of general interest to the understanding of the function of this protein. Site-directed mutagenesis of amino acids that are different in both porin sequences allowed interesting insights into the role of these amino acids in voltage dependence of eukaryotic porin.

EXPERIMENTAL PROCEDURES

PCR Amplification of D. melanogaster Porin-coding Sequences—The *CG17137* (FLYBASE, FBgn0032308; LocusID, 34499) coding sequence was amplified from a *D. melanogaster* male adult cDNA library in λgt10 using the primers DmPorin2for and DmPorin2rev (Table I). The primer DmPorin2for introduces a BamHI restriction site and the ATG start codon into the PCR product, whereas the primer DmPorin2rev introduces a HindIII restriction site and the TAA stop codon. 1.5 μl of phage lysate from the library were added to 28.5 μl of H_2O, warmed at 70 °C for 5 min, and incubated on ice. The PCR was performed according to standard protocol in a volume of 50 μl, and the final concentration of dNTPs was 0.2 mM each; the $MgCl_2$ final concentration was 1.5 mM, and the final concentration of the primers was 0.5 μM each. The PCR product was cloned into the linear TOPO-TA vector (Invitrogen) and sequenced (GenBank™ accession number AJ580916).

The control coding sequence of the DmPorin1 (GenBank™ accession number X92408) was amplified from a cDNA clone isolated in our laboratory (26). The upstream primer DmPorin1for introduced the BamHI restriction site and the ATG start codon into the PCR product; similarly, the downstream primer DmPorin1rev introduced a HindIII site together with the TAA stop codon. 10 ng of plasmid were used as template, and the reaction was performed in a volume of 50 μl. All the other conditions were the same as described above. Cloning was in the TOPO-TA vector (Invitrogen).

PCR Amplification of CG17137 from D. melanogaster Developmental Stage cDNA Libraries—The same PCR was performed to check whether the *CG17137* gene was transcribed in other developmental stages of the fly. Eight different cDNA libraries were used as templates: embryo 0–3 h; embryo 12–24 h; embryo I–II instar larval stages; early III instar larval stage; late III instar larval stage; pupal instar stage; and late pupal instar stage. The protocol for PCR and the amplification parameters were the same as described above. PCR was also designed on Rp49, a ribosomal protein, and was used to amplify the underlying sequence in the various libraries to standardize the template amount (not shown).

Cloning in the pQE30 Vector—The fragments released from the TOPO TA vector after digestion with BamHI and HindIII were ligated in the expression vector pQE30 (Qiagen) to obtain the plasmid pQE30DmPorin2. pQE30DmPorin2 also codes for an additional six histidines (His tag) upstream of the polyclonal site containing the cDNA coding for DmPorin2. XL10 gold ultracompetent cells (Stratagene) were transformed using 110 ng of pQE30DmPorin2, following the protocol provided by the manufacturer. The cells were spread on an ampicillin- and kanamycin-containing agar plate. Positive clones were picked, and the plasmid DNA was extracted from a single colony, digested, and checked on a 1% agarose gel for the right fragment size and was finally sequenced.

Site-directed Mutagenesis—The QuickChange™ site-directed mutagenesis kit (Stratagene) was used to change two glutamic acids (Glu-66 and Glu-163) into two lysines in the DmPorin2 sequence by PCR. The mutant plasmid pQE30DmPorin2e66k was obtained by using the DmPorin2 cDNA cloned in the plasmid pQE30 as template and the primers E66Kfor and E66Krev (Table I). Similarly, the mutant plasmid pQE30DmPorin2e163k was obtained by mutagenesis of pQE30DmPorin2 with the primers E163Kfor and E163Krev (Table I). A double mutant, harboring both the E66K and E163K mutations, has been created using pQE30DmPorin2e66k as template in a further PCR with the primers E163Kfor and E163Krev. The PCR was performed according to the protocol of the manufacturer with *Pfu* Turbo DNA polymerase (2.5 units) and 125 ng of the primers. The amount of template used was 10 ng. The PCR product was digested using DpnI (10 units) at 37 °C for 1 h. 1 μl of the digested DNA was transformed in competent *E. coli* M15 cells. The DNA purified from single colonies was sequenced to check the products.

Heterologous Expression of DmPorin Genes—The expression of the different genes was performed in *E. coli* strains devoid of the major porin genes (genotype: *BL21*(*DE3*), Δ*lamB ompF*::*Tn5* Δ*ompA* Δ*ompC*) to avoid possible contamination by bacterial pore-forming proteins. An overnight culture in LB medium of modified *E. coli* BL21 cells containing one of the different plasmids, pQE30DmPorin2, pQE30DmPorin2e66k, pQE30DmPorin2e163k, pQE30DmPorin2e66/163k, and pQE30DmPorin1, was diluted 1:20 in 50 ml of fresh medium and grown to an absorbance of 0.6 at 600 nm. 1 ml of culture was removed for control purposes, and isopropyl-1-thio-β-D-galactopyranoside was added to the culture in a final concentration of 1 mM to induce expression of DmPorin2 and its mutants. The cells were harvested by centrifugation after growth for another 5 h. The pellet was suspended in 8 M urea, 100 mM NaH_2PO_4, and 10 mM Tris-HCl (pH 8) and incubated for 1 h to allow the lysis of the inclusion bodies containing the recombinant proteins. The suspension in urea was centrifuged for 20 min at 10,000 rpm in an Eppendorf centrifuge, and the supernatant was collected and loaded on a nickel-nitrilotriacetic acid spin column already equilibrated with the same buffer. The column was washed with a buffer containing 8 M urea, 100 mM NaH_2PO_4, and 10 mM Tris-HCl (pH 6.3) to remove all the endogenous proteins bound to the nickel-nitrilotriacetic acid. The recombinant proteins were eluted from the column with a solution containing 8 M urea, 100 mM NaH_2PO_4, and 10 mM Tris (pH 4.5). All the samples were loaded onto a 12% SDS-PAGE to check induction and purification of the proteins.

Renaturation of the Protein—The proteins purified under denaturing conditions (8 M) have lost their structure under these conditions and needed to be refolded to get channel-forming activity in lipid bilayer experiments (29). The eluted proteins were dialyzed at 4 °C overnight against a solution containing 1% Genapol X-080 (Fluka) supplemented with 0.5% (w/v) cholesterol. The proteins were kept at 4 °C before use in the black lipid bilayer measurements.

Lipid Bilayer Experiments—The method used for the black lipid bilayer experiments has been described previously (30). Membranes were formed from a 1% (w/v) solution of diphytanoylphosphatidylcholine (Avanti Polar Lipids, Alabaster AL) in *n*-decane by painting onto a circular hole (surface area about 0.4 mm^2) separating the two compartments of a Teflon cell. For standard single channel conductance experiments, the Teflon chamber was filled with an unbuffered salt solution. The voltage across the membrane was applied through silver/silver chloride electrodes (with salt bridges) inserted into the aqueous compartments on both sides of the membrane. The membrane current was measured with a current amplifier (Keithley 427 current amplifier). The amplified signal was monitored with a storage oscilloscope and recorded on a strip chart recorder. Zero current potential measurements were performed using a high impedance electrometer (Keithley 617 current amplifier) as described previously (31).

Voltage Dependence Studies—The refolded recombinant protein was added at a concentration of 500 ng/ml to both sides of a black diphytanoylphosphatidylcholine/*n*-decane membrane. After about 20 min, the reconstitution of porin channels into the membrane reached equilibrium. Then different potentials were applied to both sides of the membrane starting with ±10 mV (data not shown). These experiments were repeated with ±20 to ±80 mV in steps of ±10 mV.

The experiments were analyzed in the following way. The membrane conductance (G) as a function of voltage, V_m, was measured when the opening and closing of channels reached an equilibrium, *i.e.* after the exponential decay of the membrane current following the voltage step V_m. G was divided by the initial value of the conductance (G_0, which was a linear function of the voltage) obtained immediately after the onset of the voltage.

Ion Selectivity Determination—Diphytanoylphosphatidylcholine membranes were formed in 50 mM KCl, and concentrated porin solutions were added to the aqueous phase when the membranes were in the black state. After incorporation of 10–100 channels into a membrane, salt gradients were established by addition of small amounts of concentrated KCl solution to one side of the membrane. Analysis of the zero current membrane potential was performed using the Goldman-Hodgkin-Katz equation (31).

Three-dimensional Modeling of the DmPorin2 Amino Acid Sequence—The three-dimensional model of DmPorin2 was computed by using a procedure similar to that adopted previously for porin (isoform 1 or VDAC1) (7). In summary, the protein topography starting from the sequence was predicted with a neural network-based method and a hidden Markov model-based method (32, 33).

Both predictors suggested 16 transmembrane strands along the protein sequence, with the same reliability index. An α-helix predictor detected a putative α-helical segment in the N-terminal portion of the chain (residues 7–20, data not shown), similar to what was reported previously (7) for VDAC1.

The target was then modeled on templates taken from the Protein Data Bank (codes 2OMF, 1PRN, and 2POR) with the same number of strands in the barrel. MODELLER 6.2 (34) was used to perform essentially a building by homology procedure. The N-terminal portion was not considered, since the expert-driven alignment between the target and the templates is strictly based on the transmembrane regions, constraining gaps in the loop regions, external to the membrane phase. Small differences in the alignment led to slightly different models; the model with the best PROCHECK (35) score (minimum percentage of residues in disallowed conformation) was considered as the most reliable model.

RESULTS

Expression and Purification of DmPorin1 and DmPorin2—Fig. 1*A* shows an agarose gel of the PCR amplification from the *D. melanogaster* male adult cDNA library using the primers DmPorin2for and reverse specific for *CG17137* (DmPorin2) and the primers DmPorin1for and reverse specific for porin (DmPorin1). Both cDNAs were cloned in a TA vector and then in the pQE30 expression vector to yield the plasmids pQE30DmPorin1 and pQE30DmPorin2. Both plasmids were transferred into an *E. coli* BL21 strain, which lacks all major outer membrane proteins. The expressed proteins were purified from inclusion bodies. They had the expected apparent molecular masses (Fig. 1*B*). The expression level in bacteria was around 30 μg of protein/ml of bacterial culture for both proteins, and the purified protein was free of bacterial contaminants as judged by SDS-PAGE.

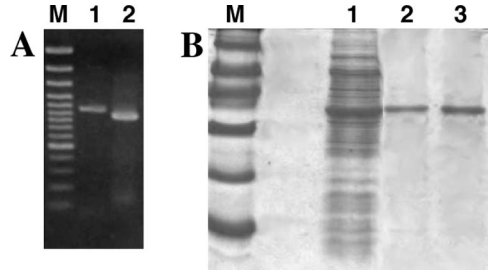

FIG. 1. **Amplification of porin coding sequences from a *D. melanogaster* adult cDNA library and purification of the protein expressed in bacteria.** *A*, agarose gel showing the PCR product obtained by amplification of the coding sequence corresponding to DmPorin2 (*lane 1*) and to DmPorin1 (*lane 2*). *M* indicates GeneRuler 100-bp ladder (Fermentas). *B*, expression of DmPorin2 in *E. coli* BL21. SDS-PAGE of the bacterial lysate obtained after isopropyl-1-thio-β-D-galactopyranoside induction (*lane 1*) and of the fractions eluted from a nickel tag spin column after washing the unretained proteins (*lanes 2* and *3*). The purified protein is shown. *Lane M* shows low molecular weight markers from Amersham Biosciences; the molecular masses are 14.4, 21.5, 31, 45, 66.2, and 97.4 kDa from the *bottom* to the *top*. The gel was stained with Coomassie Blue.

PCR Amplification of the Gene for DmPorin2 in Different RNA-derived cDNA Libraries—A similar PCR amplification was performed on a set of *D. melanogaster* libraries derived by poly(A)$^+$ RNA purified at different developmental stages of the fly (Fig. 2). The coding sequence delimited by the PCR primers (DmPorin2for and rev) was specifically amplified in all the libraries tested and had a similar level. This result demonstrates that the gene of DmPorin2 was transcribed in all developmental stages of *D. melanogaster*.

Channel Forming Activity and Voltage Dependence of the Recombinant Porins—The electrophysiological properties of the purified and refolded DmPorin1 were studied in black lipid bilayer membranes and compared with those of the native porin purified from *D. melanogaster* mitochondria, which have been reported previously (36). Single channel experiments performed with small amounts of refolded recombinant protein (1–10 ng/ml) in 1 M KCl showed current increases in discrete steps with a predominant single channel conductance of about 4.5 nS (Fig. 3), similar to native porin isolated from *D. melanogaster* mitochondria. This result also indicated that the His tag attached to the recombinant protein did not interfere with the channel conductance.

In the next step the voltage dependence of DmPorin1 was analyzed. Voltage dependence is a typical feature of mitochondrial porins (3). Starting with application of about 20–30 mV, the membrane current decreased in an exponential fashion for both positive and negative potentials. This result indicated symmetrical response to the applied voltage. The data of Fig. 3*D* show the plot of the relative conductance G/G_0 as a function of the applied voltage, *i.e.* it corresponds to the voltage dependence of DmPorin1. It is noteworthy that the voltage dependence of the recombinant porin was identical to that measured previously with the native mitochondrial porin of *D. melanogaster* (36). These results indicate that recombinant DmPorin1 was successfully re-natured and that its properties were undistinguishable from those of native porin.

Similar experiments were performed with the refolded recombinant DmPorin2. It also formed channels in lipid bilayer membranes with a long lifetime at low voltage. DmPorin2 also formed preferentially channels with a single channel conductance of 4.5 nS in 1 M KCl (Fig. 3*A*). However, the channel forming activity was much smaller than that of DmPorin1 under otherwise identical conditions. At least a 10 times higher

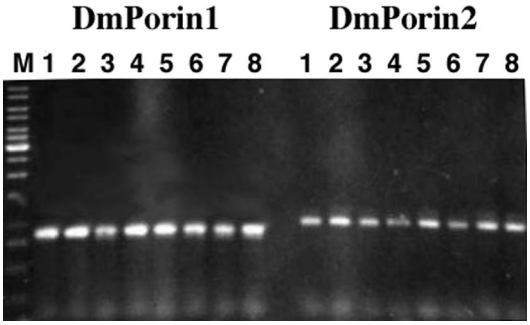

FIG. 2. **Amplification of the DNA sequence corresponding to the DmPorin1 and DmPorin2 coding sequences in cDNA libraries of various developmental stages of the fly.** Amplification performed on cDNA libraries. *Lane 1,* embryo 0–3 h; *lane 2,* embryo 12–24 h; *lane 3,* embryo; *lane 4*, I–II instar larval stage; *lane 5,* early III instar larval stage; *lane 6,* late III instar larval stage; *lane 7,* early pupal instar stage; *lane 8,* late pupal instar stage. *Lane M,* bp ladder. The amplification in the adult is shown in Fig. 1.

concentration of DmPorin2 was needed to obtain the same number of reconstituted channels as compared with DmPorin1. It is unknown whether this is a normal feature of DmPorin2 or is because of an incomplete refolding process. The results of single channel experiments suggest that both porins form channels with very similar properties (Fig. 3). The voltage dependence of DmPorin2 was also investigated and compared with that of DmPorin1. Most surprising, DmPorin2 did not display any voltage dependence as clearly evidenced by Fig. 3, *B* and *D*.

The single channel conductance of DmPorin1 and DmPorin2 in different salt solutions was also studied. The recombinant DmPorin1 and DmPorin2 proteins formed channels that had the same conductance only in 1 M KCl. However, in other salt solutions with different mobility of the ions the conductance differed. When the salt was composed of the highly mobile Cl^- anion and the less mobile Li^+ cation, the conductance of DmPorin1 was about twice that recorded with DmPorin2. The opposite was the case when a less mobile anion (CH_3COO^-) was combined with a mobile cation (K^+). This influence on the conductance could be explained by the effect of charges influencing ion transport through the channel, *i.e.* DmPorin1 and DmPorin2 could exhibit different ion selectivity (Fig. 3*C*).

This possibility was studied by zero current membrane potential measurements, which allow the calculation of the permeability ratio P_{cation} divided by P_{anion} (P_c/P_a) for channels in multichannel experiments. In all experiments with DmPorin1, the more diluted side of the membrane became negative, which indicated preferential movement of anions through the channel. The zero current membrane potential for a 4-fold gradient of KCl was about −2.2 mV (average of three experiments) on the more diluted side (Table II). The results were in good agreement with data derived previously from native porin isolated from mitochondria (36). Similar experiments were also performed with recombinant DmPorin2. This channel was found to be cation-selective because the more dilute side had a positive potential of about 18 mV for an 8-fold KCl gradient, and the permeability coefficient P_{cation} divided by P_{anion} was 3.8. This result indicated that the two isoforms of *D. melanogaster* porins exhibit different ion selectivity.

Electrophysiological Properties of Three DmPorin2 Mutants—The data from the reconstitution experiments clearly demonstrated a different functional behavior of the two porins. Analysis of the amino acid composition and sequence comparison of the two proteins supported this view. DmPorin1 has a smaller number of acidic amino acids (in particular, 11 glutamic acids *versus* 22 in DmPorin2) and a similar number of basic amino acids (25 lysines and 4 arginines against 21 and 7, respectively, in DmPorin2). The alignment of DmPorin1 and DmPorin2 showed that 10 lysines are in a conserved position, but four lysines in DmPorin1 are exchanged by glutamic acids in DmPorin2 (Fig. 4). In particular lysines 135 and 176 (sequence position of DmPorin2) are not well conserved among porins from different sources (see Fig. 4) and were excluded from our study. We thus selected glutamic acid 66 and 163 in DmPorin2, corresponding to Lys-65 and Lys-162 in DmPorin1, for site-directed mutagenesis to study the role of these amino acids in voltage dependence and ion selectivity.

To check whether Glu-66 and Glu-163 may influence the ion selectivity and the voltage dependence of DmPorin2, they were replaced by lysines through site-directed mutagenesis. The single mutants E66K and E163K were constructed together with the double mutant E66K/E163K. The electrophysiological properties of the three mutant proteins were tested in lipid bilayer experiments. The mutations resulted in dramatic changes of the functional behavior of the protein.

The mutant E66K had a higher channel forming activity in low salt solutions possibly because the ionic strength interfered with the protein stability; thus, decreasing the ionic strength from 1 M to 150 mM or 50 mM KCl resulted in well defined channels (Fig. 5). In 1 M KCl it behaved in a manner similar to DmPorin2. The most frequent single channel conductance was 4 nS; nevertheless, the conductance was very disturbed, and the incorporation events were scarce.

Channel forming activity of E163K was lower than that of DmPorin2, and the single channels showed higher current noise even in low ionic strength buffer (data not shown). The most frequent single channel conductance in 1 M KCl was around 2.5–3 nS (Fig. 5). The smaller channel forming activity of the mutant as compared with DmPorin2 may be caused by the exchange of Glu-163 by lysine, which seems to interfere with the stability of the protein.

Similar results were obtained for the double mutant, E66K/E163K, which also showed high current noise in the reconstitution experiments. The most frequent single channel conductance was 2.5–3 nS in 1 M KCl (Fig. 5). The behavior of this protein could be explained if the role of both glutamic acids is taken into consideration. Glutamic acid 66 mutation did not alter channel forming activity, but the substitution of glutamate 163 by lysine did. The double mutant showed the characteristic of the two single amino acid replacements and in particular that of the E163K mutant.

The Replacement of Glutamic Acids by Lysines Confers Voltage Dependence to rDmPorin2—DmPorin1 has a net positive charge and is voltage-dependent, whereas DmPorin2 has a net negative charge and is voltage-independent. Since it has been proposed that the voltage sensor of VDAC is formed by a cluster of positively charged groups (37), it is possible that the amino acids Lys-66 and Lys-163 of DmPorin1 may be involved in the voltage sensor. This means that the presence of glutamic acid residues at the same position in DmPorin2 can explain, at least in part, the absence of any voltage dependence in this protein. To check this possibility, the voltage dependence of DmPorin2 mutants was investigated. The ratio G/G_0 calculated for DmPorin2 E66K was 0.89 at a voltage of ± 50 mV and 0.8 at a voltage of ± 80 mV, which suggests that this mutant exhibited some voltage dependence. Similar results were obtained with the other mutants, which were also slightly voltage-dependent (see Table II and Fig. 5). It is noteworthy that the double mutant E66K/E163K exhibited the highest voltage dependence (G/G_0 was 0.61 at ± 50 mV and 0.58 at ± 80 mV), modifying the voltage independence shown for DmPorin2.

Besides the voltage dependence we also studied the ion se-

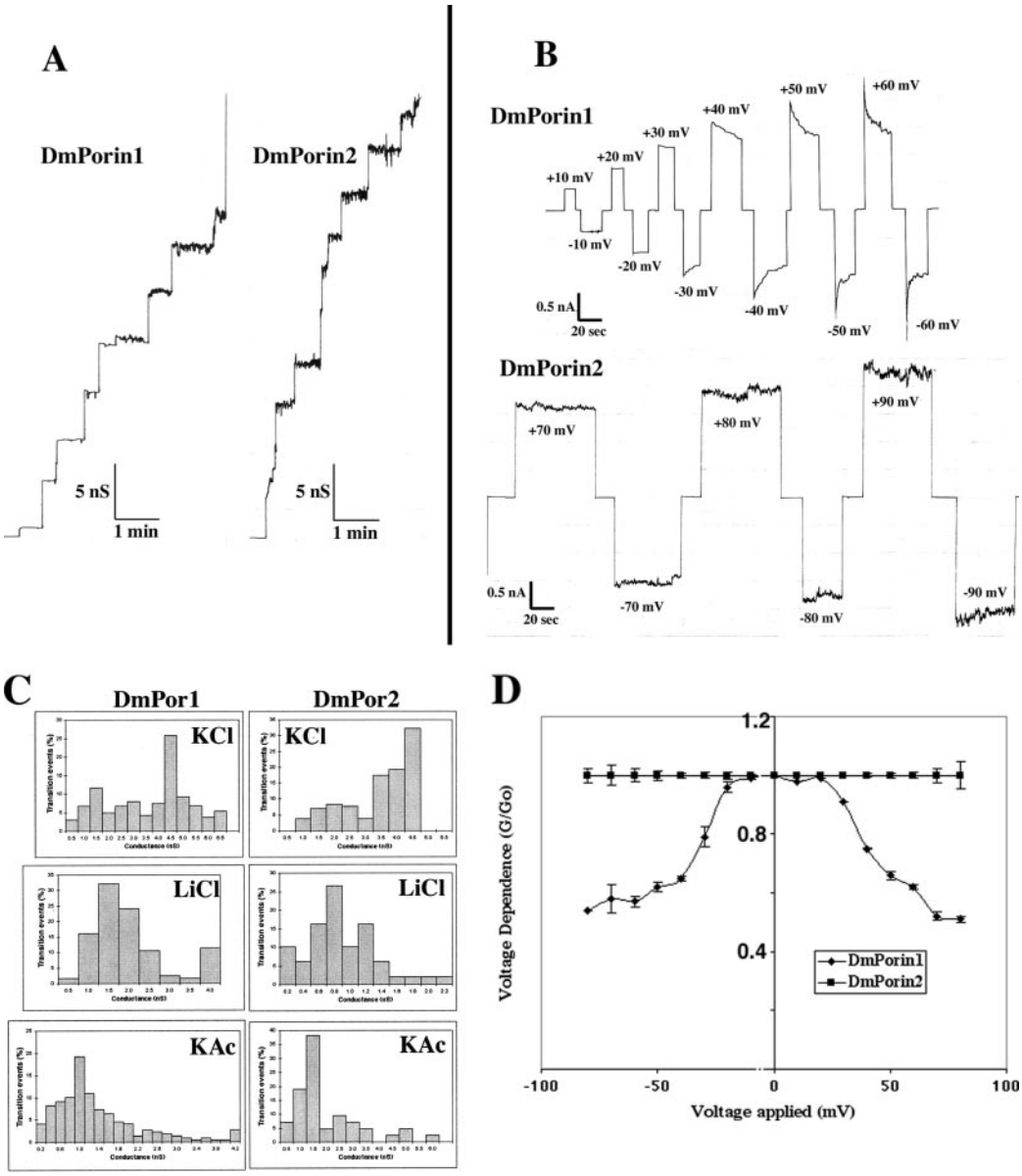

FIG. 3. **Electrophysiological features of recombinant DmPorin1 and DmPorin2.** *A*, stepwise increase of the membrane current (expressed as nS) after the addition of recombinant DmPorin1 and DmPorin2 to a black lipid bilayer membrane given as a function of time. The aqueous phase contained 10 ng/ml protein (DmPorin1) or 100 ng/ml protein (DmPorin2) and 1 M KCl. The membrane was formed from diphytanoylphosphatidylcholine/*n*-decane. The voltage applied was 10 mV; $T = 20$ °C. *B*, voltage dependence of DmPorin1 and DmPorin2 in multichannel experiments. The porin pores were incorporated in a membrane from 1% diphytanoylphosphatidylcholine/*n*-decane. The voltage across the membrane was switched to the positive and negative values indicated upon the traces. The aqueous phase contained 1 M KCl, pH 6; $T = 20$ °C. *C*, *histograms* of the probability for the occurrence of a given conductivity unit observed with membranes formed of diphytanoylphosphatidylcholine/*n*-decane in the presence of DmPorin1 and DmPorin2. In the *y* axis is the % of transition events with a given conductance increment observed in the single-channel experiments. It was calculated by dividing the number of transitions with a given conductance increment by the total number of conductance transitions. The voltage applied was 10 mV and $T = 20$ °C. The aqueous phase contained from the top 1 M KCl, 1 M LiCl, and 1 M KCH_3COOH. One hundred to two hundred events were recorded for each histogram. *D*, voltage dependence G/G_0 comparison of DmPorin1 and DmPorin2. Voltage dependence G/G_0 was a function of the applied voltage. Points were mean ± S.E. DmPorin2 is not voltage-dependent.

lectivity of the mutants. This was done to check whether the amino acids Glu-66 and Glu-163 were also involved in channel selectivity. The results are also included in Table II. The ratio P_c/P_a derived for DmPorin2 E66K was similar (indeed even higher) than that of DmPorin2, which means that this protein also formed cation-selective channels. The ratio P_c/P_a derived for the E163K and the E66K/E163K mutants in KCl was about 1.2 in both cases, *i.e.* the channels formed by these proteins were still slightly cation-selective although much less than DmPorin2 ($P_c/P_a = 3.8$). These results suggest that the mutation E66K has no effect on ion selectivity. The mutation E163K had a higher impact on the selectivity of the channels, but they were still cation-selective, which means that other charged amino acids also have an influence on the ion selectivity of these channels.

Predicting the Structure of the DmPorin2 Pore—The DmPorin2 coding sequence (GenBank™ accession number AJ580916) obtained by amplification of an adult cDNA library was identical to the sequence deduced from the genome. In comparison with the other fruit fly porins, DmPorin2 contains an additional 10 amino acids (EQQGGDVVVN) toward the C terminus (Fig. 4).

The sequence of DmPorin2 was modeled by a refined neural network tool to predict a secondary structure and, in turn, a

TABLE II

Single channel conductance, ion selectivity, and voltage dependence of native, recombinant, and mutant D. melanogaster porins

	Single channel conductance[a]	Reversal potential	Ion selectivity	Voltage dependence	Gating charge
	nS	*mV*	P_c/P_a[b]	G''/G_0[c]	n[d]
Native DmPorin1[e]	4.5		0.63	0.70	
rDmPorin1[f]	4.5	−2.2 ± 0.1	0.82 ± 0.05	0.64 ± 0.01	1
rDmPorin2	4.5	18.5 ± 0.6	3.80 ± 1.00	1.0 ± 0.01	0
rDmPorin2 E66K	0.6[g]	24.7 ± 0.3	4.50 ± 0.60	0.89 ± 0.02	∼0
rDmPorin2 E163K	2.5	3.5 ± 0.2	1.20 ± 0.20	0.87 ± 0.06	∼1.4
rDmPorin2 E66/163K	2.5	2.1 ± 0.2	1.20 ± 0.30	0.61 ± 0.01	∼1.6

[a] The applied voltage was 10 mV and the temperature 20 °C. The single channel value reported in the table is the most frequent conductance value in 1 M KCl (see also Figs. 3 and 5). At least 200 reconstitution events were examined for each protein.

[b] P_c/P_a is the ratio of the permeability of cations versus anions calculated using the Goldman-Hodgkin-Katz equation (31). The reported values represent the mean of the experimental data with standard deviation.

[c] The voltage dependence is the ratio between the membrane conductance at ±50 mV and the stationary conductance G_0. The data are mean ± S.E.

[d] The gating charge n was calculated according to Ref. 1.

[e] Data taken from Ref. 36.

[f] The prefix r indicates recombinant protein.

[g] The most frequent single channel conductance in 0.15 M KCl.

```
            1                                                   50                66                                  100
DmPorin2    .......... .MAAKTPTYP DLGKLARDLF KRG.YHPGIW QIDCKTLTNS GIEFFTTGFA SQDNSKVTGS LQSKYKIEDQ GLTLTERWNT ENWLFGEIMH
DmPorin1    .......... ...MAPPSYS DLGKQARDIF SKG.YNFGLW KLDLKTKTSS GIEFNTAGHS NQESGKVFGS LETKYKVKDY GLTLTEKWNT DNTLFTEVAV
POR1_MOUSE  .......... ...AVPPTYA DLGKSARDVF TKG.YGFGLI KLDLKTKSEN GLEFTSSGSA NTETTKVNGS LETKYRWTEY GLTFTEKWNT DNTLGTEITV
POR2_MOUSE  MAECCVPVCP RPMCIPPPYA DLGKAARDIF NKG.FGFGLV KLDVKTKSCS GVEFSTSGSS NTDTGKVSGT LETKYKWCEY GLTFTEKWNT DNTLGTEIAI
POR3_MOUSE  .......... ..MCNTPTYC DLGKAAKDVF NKG.YGFGMV KIDLKTKSCS GVEFSTSGHA YTDTGKASGN LETKYKVCNY GLTFTQKWNT DNTLGTEISW
POR1_YEAST  .......... ...MSPPVYS DISRNINDLL NKDFYHATPA AFDVQTTTAN GIKFSLKAKQ PVKDGPLSTN VEAKLNDKQT GLGLTQGWSN TNNLQTKLEF
POR2_YEAST  .......... ...MALRFFN DISRDVNGLF NRDFFHTNPL SLNISTTTEN GVNFTLKAKQ GVTEGPIQTS VEGRFYDRKE GVSLSQSWSN QNRLNTRIEF
Conserved                         D                          T     G  F                             G      W    N L

           101                                             135                 163           176
DmPorin2    RDKLAQGLML AVEAKFQPGS NEADGKFKMG YAQDNFNFLA DIGLNSE PI LNCSLVVGHK EFLGGVGTEF DVGNTELKGW KVALGWTNET ATLHGELKNG
DmPorin1    QDQLLEGLKL SLEGNFAPQS GNKNGKFKVA YGHENVKADS DVNIDLKGPL INASAVLGYQ GWLAGYQTAF DTQQSKLTTN NFALGYTTKD FVLHTAVNDG
POR1_MOUSE  EDQLARGLKL TFDSSFSPNT GKKNAKIKTG YKREHINLGC DVDFDIAGPS IRGALVLGYE GWLAGYQMNF ETSKSRVTQS NFAVGYKTDE FQLHTNVNDG
POR2_MOUSE  EDQICQGLKL TFDTTFSPNT GKKSGKIKSA YKRECINLGC DVDFDFAGPA IHGSAVFGYE GWLAGYQMTF DSAKSKLTRS NFAVGYRTGD FQLHTNVNNG
POR3_MOUSE  ENKLAEGLKL TLDTIFVPNT GKKSGKLKAS YRRDCFSLGS NVDIDFSGPT IYGWAVLAFE GWLAGYQMSF DTAKSKLSQN NFALGYKAAD FQLHTHVNDG
POR1_YEAST  A.NLTPGLKN ELITSLTPGV .AKSAVLNTT FTEPFFTARG AFDLCLKSPT FVGDLTMAHE GIVGGAEFGY DISAGSISRY AMALSYFAKD YSLGATLNNE
POR2_YEAST  S.KIAPGWKG DVNAFLTPQS .IKNAKFNLS YAQKSFAART SIDI.LQPKD FVGSVTLGHR GFVGGTDIAY DTAAGLCARY AMSIGYLARE YSFILSTNNR
Conserved         G           P                                                              G      D

            201                                                250                                                    300    306
DmPorin2    DTWLASLFYK ASEKIDAGIE VTKGAGGGEA AEGEQQGGDV VVNLGMIYHL EEDALVRAKV NNLVELGLGY EQKLRDGITA SISAVLDCNN FKDGNHRFGV GIALQC
DmPorin1    QEFSGSIFQR TSDKLDVGVQ LSWASGTSNT KFA....... ...IGAKYQL DDDASVRAKV NNASQVGLGY QQKLRDGVTL TLSTLVDGKN FNAGGHKIGV GLELEA
POR1_MOUSE  TEFGGSIYQK VNKKLETAVN LAWTAGNSNT RFG....... ...IAAKYQV DPDACFSAKV NNSSLIGLGY TQTLKPGIKL TLSALLDGKN VNAGGHKLGL GLEFQA
POR2_MOUSE  TEFGGSIYQK VCEDFDTSVN LAWTSGTNCT RFG....... ...IAAKYQL DPTASISAKV NNSSLIGVGY TQTLRPGVKL TLSALVDGKS FNAGGHKLGL ALELEA
POR3_MOUSE  TEFGGSIYQK VNERIETSIN LAWTAGSNNT RFG....... ...IAAKYKL DCRTSLSAKV NNASLIGLGY TQTLRPGVKL TLSALIDGKN FNAGGHKVGL GFELEA
POR1_YEAST  QITTVDFFQN VNAFLQVGAK ATMNCKLPNS NVN....... .IEFATRYLP DASSQVKAKV SDSGIVTLAY KQLLRPGVTL GVGSSFDALK LSEPVHKLGW SLSFDA
POR2_YEAST  QCATASFFQN VNRYLQVGTK ATLQSKT.SS NMN....... .IEFVTRYVP DSISQVKAKI ADSGLTTLSY KRNLNKDISL GVGMSFNALQ LTEPVHKFGW SLSFSP
Conserved                                                                 Y          AK         L Y    L                       H
```

FIG. 4. **Sequence comparison of representative porin proteins.** Eukaryotic porin isoforms were aligned by Clustal. The proteins aligned to the two DmPorins are isoform families from lower (*S. cerevisiae* yeast, POR1_YEAST, Swiss-Prot accession number P04840, POR2_YEAST, Swiss-Prot accession number P40478) or higher eukaryotes (mouse, POR1_MOUSE, Swiss-Prot accession number Q60932, POR2_MOUSE, Swiss-Prot accession number Q60930, POR3_MOUSE, Swiss-Prot accession number Q60931, DmPorin 1, Swiss-Prot accession number Q94920). Highlighted are positions 66, 135, 163, and 175 where lysine is substituted with glutamic acid in the DmPorin2.

three-dimensional model of the protein (Fig. 6). The neural network software indicates the presence of 16 amphipathic β-strands and thus the compatibility of the sequence with the general 16 β-strands and β-barrel model published in a previous work (7). This is a predictive analysis that should wait for confirmation by true crystallized structures. It is nonetheless interesting because it suggests that the location of the glutamic acids subjected to mutagenesis might be of relevance for the electrophysiological results. In such a predictive model Glu-66 is located in a turn connecting β-strands 3 and 4, whereas Glu-163 is predicted in the β-strand 9. These amino acids are thus predicted on the opposite sides of the channel, with their residues protruding inside the pore or just outside it (Fig. 6). The 9-amino acid additional stretch is located in strand 7.

DISCUSSION

Porin/VDAC is a multifunctional protein involved in several cellular processes, but its biological significance has not been fully characterized (38–42). In particular, the functions of the various VDACs or eukaryotic porins and their relationships are still open questions. In recent years we used the *D. melanogaster* to elucidate some of these questions in a relatively simple eukaryotic model organism (26–28, 36). More porin genes have been found in the fly after the completion of the fly genome project (28). No information is available about the possible expression of the encoded proteins or the specific function of the gene products. Very recently Komarov *et al.* (43) have reported on the physiological properties of *D. melanogaster* porin-like proteins.

In the present work we have shown for the first time that the transcript corresponding to the gene termed *CG17137* (28) is widely present in the various developmental stages of the fly. We have thus amplified its coding sequence from a cDNA library and expressed it in *E. coli*. After refolding in the presence of sterol, lipids, and detergents (29, 44), this protein was used for reconstitution experiments in black lipid membrane and showed pore forming activity. The formation of channels by DmPorin2 exhibited features typical for eukaryotic porins. Secondary structure predictions suggest the presence of amphipathic β-strands (45), and its sequence could be folded according to a three-dimensional model published previously (7).

A detailed analysis of the channel formation of DmPorin2 revealed interesting differences between the homologous Dm-

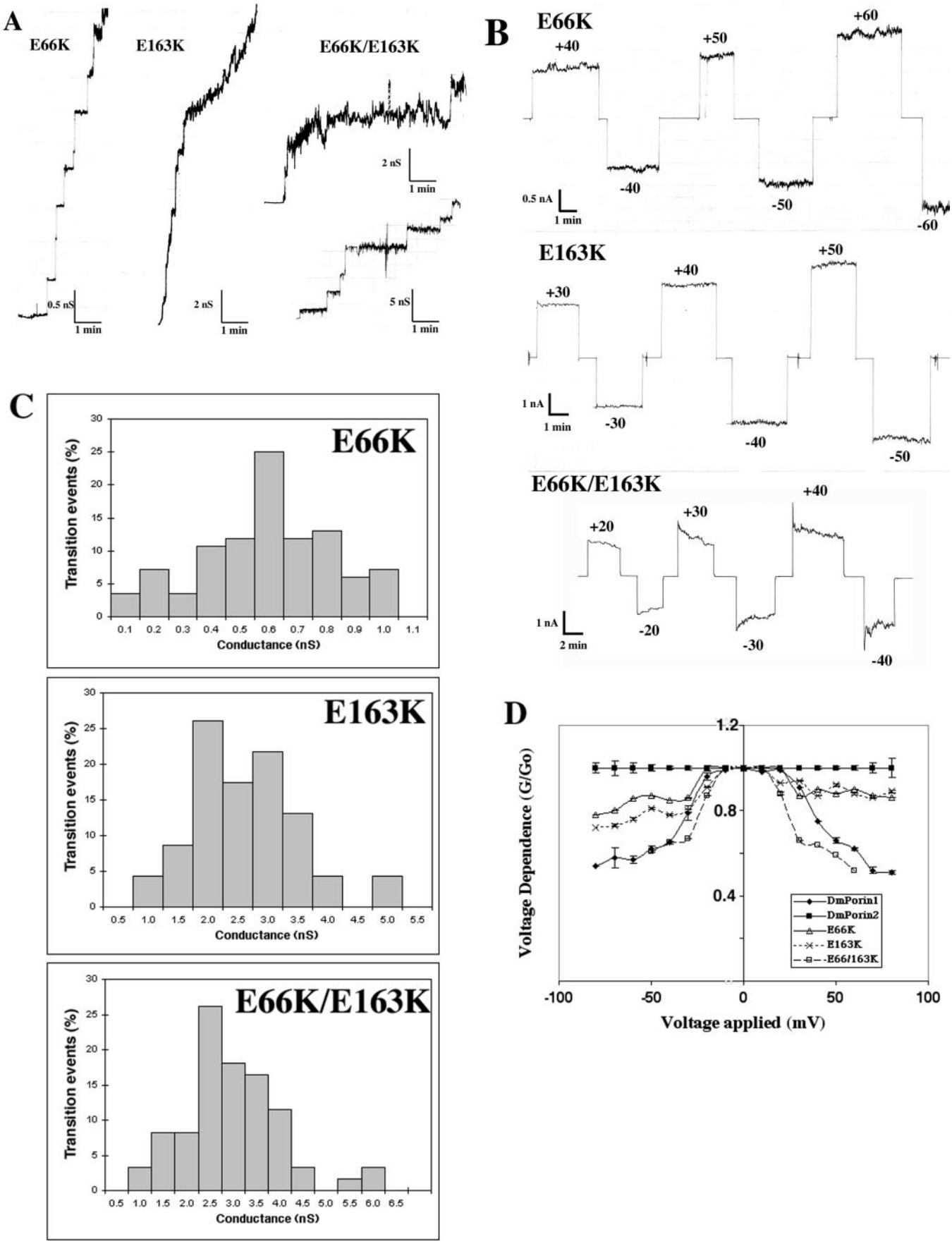

FIG. 5. **Electrophysiological features of DmPorin2 mutants.** *A*, stepwise increase of the membrane current (expressed as nS) after the addition of recombinant DmPorin2 mutants to a black lipid bilayer membrane given as a function of time. The aqueous phase contained 100 ng/ml protein of the various recombinant porins. Salt concentration was 0.150 M KCl (E66K) or 1 M KCl (E163K and E66K/E163K). Two traces are reported for the mutant E66K/E163K to show the noise level in such records. The membrane was formed from diphytanoylphosphatidylcholine/

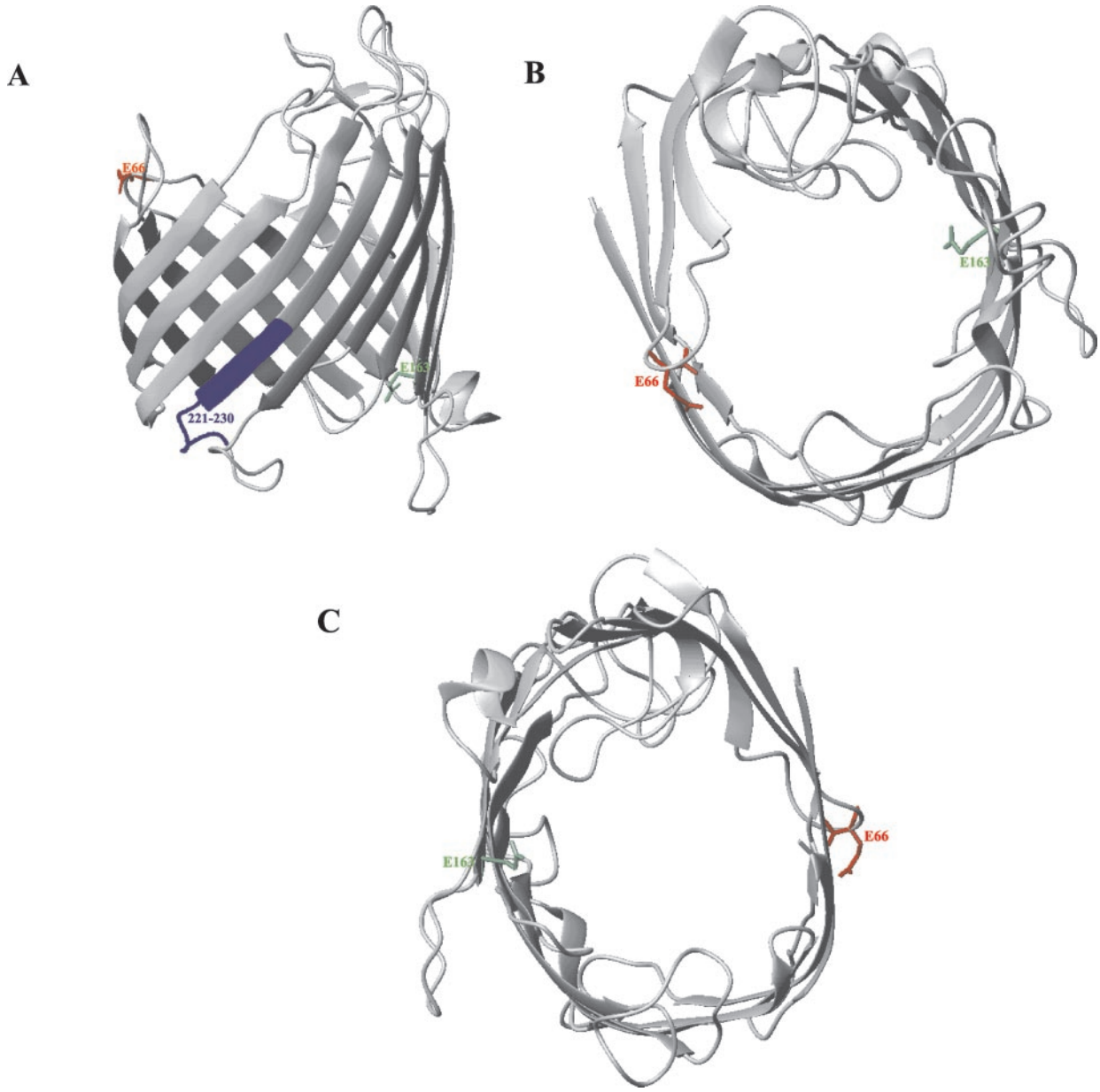

FIG. 6. **Three-dimensional model of DmPorin2.** The glutamic acid Glu-66 is highlighted in *red*, and the glutamic acid Glu-163 is in *green*. The position of the amino acid stretch 221–230, which represents an amino acid addition peculiar of this gene, is indicated in *blue*. *A*, frontal view; *B*, view from a hydrophilic side; *C*, view from opposite hydrophilic side.

Porin1 protein. DmPorin2 is cation-selective instead of anion-selective protein, and it is voltage-independent. The charged amino acid residues in or near the channel wall are likely responsible for the weak ion selectivity of the channels formed by the eukaryotic porins (46). It is clear from the amino acid composition that DmPorin2 is a more acidic protein than DmPorin1. We assumed that exchanges of oppositely charged amino acids could cause the change of electrophysiological properties of the protein. We selected Glu-66 and Glu-163 in DmPorin2, corresponding to Lys-65 and Lys-162 in DmPorin1, for a site-directed mutagenesis study by assuming these amino acids were relevant for voltage dependence and ion selectivity. The E66K mutation did not change the preference for cations of the protein, whereas E163K decreased the cation selectivity. Neither E163K nor the double mutant E66K/E163K were able to completely reverse the ion selectivity to anion-selective as in

n-decane. The voltage applied was 10 mV; $T = 20$ °C. *B*, voltage dependence of DmPorin2 mutants in multichannel experiments. The porin pores were incorporated in a membrane from 1% diphytanoylphosphatidylcholine/*n*-decane. The voltage across the membrane was switched to the positive and negative values indicated on the traces. The aqueous phase contained 1 M KCl, pH 6; $T = 20$ °C. *C*, *histograms* of the probability for the occurrence of a given conductivity unit observed with membranes formed of diphytanoylphosphatidylcholine/*n*-decane in the presence of DmPorin2 mutants. In the *y* axis the % of transition events with a given conductance increment is observed in the single channel experiments. It was calculated by dividing the number of transitions with a given conductance increment by the total number of conductance transitions. The voltage applied was 10 mV, and $T = 20$ °C. The aqueous phase contained 1 M KCl with the exception of E66K, analyzed in 0.150 M KCl. One-two hundred events were recorded for each histogram. *D*, voltage dependence G/G_0 comparison of DmPorin1 and DmPorin2 with DmPorin2 mutants. Points were mean ± S.E. Voltage-dependent G/G_0 was a function of the applied voltage. Each point is the ratio between the membrane conductance (G) at the indicated voltage and the initial value of the conductance (G_0, which was a linear function of the voltage) obtained immediately after the onset of the voltage. Points were mean ± S.E.

DmPorin1. It clearly has to be concluded that only the position corresponding to Glu-163 is relevant in the ion selectivity.

Voltage dependence of the mutants was studied next. As described above, DmPorin2 did not show any voltage dependence, which is a typical feature of these proteins, consequently also called VDAC. Most surprising, both single mutations E66K and E163K added a slight voltage dependence to DmPorin2. The double mutant E66K/E163K showed a higher voltage dependence, comparable in size to the voltage dependence measured for recombinant DmPorin1. It is tempting to speculate that the effect of the charge changes at positions 66 and 163 was additive.

For additional hints regarding the reason for such evident influence exerted by these mutations on the electrophysiological properties of the DmPorin2, we used predictive modeling (7) to speculate about the position of residues Glu-66 and Glu-163 in the protein. Most interesting, in our model both residues 66 and 163 are located close to the predicted rim of the pore, on opposite sides. In particular, residue 66 is located in a tight turn between two β-strands, whereas residue 163 is at the end of a β-strand, with the amino acid side group protruding into the channel (Fig. 6). The positions predicted for these two residues would make them good candidates for a crucial role in the voltage dependence and selectivity of the channel.

We examined the literature to compare these results with other predictive models reported by other groups. In a 12 β-strand model for the yeast VDAC (46), the Lys-65 residue, corresponding to the protein alignment (Fig. 4) to Lys-65 in DmPorin1 and to Glu-66 in DmPorin2, is also predicted at the end of a β-turn (46), exactly as in our model. Blachly-Dyson *et al.* (46) mutated Lys-65 in glutamic acid, and they investigated the influence of this mutation on the ion selectivity. A slight effect was noticed; nevertheless, the change in reversal of potential was rather small (46). Also in a 13 β-strand model designed for the *Neurospora crassa* mitochondrial porin, this conserved lysine was fixed in the same context, *i.e.* at the tip of a β-strand in a very tight turn; but its contribution to the functional features of the protein was not studied (6). Glu-163 is not conserved in yeast or *N. crassa*; thus it was never mutated before. Most interesting, the residues corresponding to positions 65 and 163 are located on opposite sides of the membrane in the more recent *N. crassa* model (6). However, they are on the same side of the membrane in the very obsolete yeast model (46).

Voltage dependence is a typical feature of eukaryotic porins; it has the effect of partially closing the pore. Even though it has been detected only in reconstituted systems, it is thought to be of metabolic significance. Metabolites such as succinate, malate, and ATP, normally able to diffuse through the pore, should be delayed in crossing the outer membrane after the voltage-dependent switching of porin to closed states (9, 10).

Thomas *et al.* (37) proposed that a positively charged domain in the pore-forming protein, called voltage-sensor, distributed over a large region of the protein should be responsible for sensing voltage changes across the membrane and thus for triggering the partial closure of the pore. If this is correct, increasing the positive charge in the voltage sensor should increase the voltage dependence as it has indeed been demonstrated by the same authors with the substitution of lysine for glutamate at the end of a putative transmembrane β-strand in yeast (E145K and E152K) (47). In the present study the glutamic acids mutated to lysines are also localized at the end of transmembrane strands, thus suggesting that the voltage sensor may also involve amino acid residues located at the rim of the pore.

In a very recent report Komarov *et al.* (43) expressed in Δ*porin1* yeast cells three porin-like proteins corresponding to the genes *CG17137*, *CG17139,* and *CG17140*. In agreement with our work they found that the protein corresponding to the *CG17137* gene shares with DmPorin1 many properties common to eukaryotic porins. When expressed in the Δ*porin1* yeast strain, protein *CG17137* was able to complement the cellular growth defect, allowing growth on glycerol at 37 °C. Upon incorporation in planar phospholipid bilayers, this protein formed typical channels even though the authors found conducting events ranging between 0.5 and 8 nS in 1 M NaCl. Upon reconstitution in artificial vesicles, this protein allowed permeation of non-electrolytes with a molecular mass of 3,800 Da but not of 6,800 Da. This means that *CG17137* or DmPorin2 permits the permeation of hydrated molecules with the same exclusion limits typical of porin channels. They did not reach any conclusion concerning the channel ionic selectivity, because they observed that sometimes the closure of the channel resulted in a change toward preference for cations, but at other times the opposite was true (43). In contrast to our work, Komarov *et al.* (43) showed a plot where a membrane containing 2–4 channels showed voltage-dependent closure. Surprisingly there are no statistics for any of the data presented in the paper, and thus the voltage dependence reported might be related to random events.

In conclusion, DmPorin2 shares with DmPorin1 the ability to form channels with a relatively large diameter. From electrophysiological studies we can conclude that they should have a similar function in the cell. This is confirmed by the pattern of transcription and by the structural predictions. The different although slight ionic selectivity might be related to a subtle preference for metabolites. More important could be the difference in voltage dependence. A porin not responding to changes in the voltage across the membrane could give rise to a non-regulated channel, always open to hydrophilic permeation. A similar channel-forming protein might thus be useful in emergency situations to rescue damages or impairment to the main porin DmPorin1. Ultimately, we cannot exclude the possibility that the second isoform might be involved in other different functions not yet known. For example, a recent report (20) suggests a primary function of VDAC2 isoform in mammals as a specific inhibitor of BAK-dependent mitochondrial apoptosis.

We did not characterize the other predicted genes *CG17139* and *CG17140* from *D. melanogaster* because there is not a general consensus about their relevance. The compilators of the Berkeley *Drosophila* Genome Project have recently joined these two putative genes in a single one (*CG31722*). In comparison with the other predicted porin-like genes, DmPorin2 (or *CG17137*) has a higher probability to be a true gene, since it also contains a putative promoter and other untranslated regions. In addition, there is a large number of reported EST sequences related to it in the data bases, and its sequence does not show the unexpected 60-amino acid long N-terminal addition predicted for the putative products of genes *CG17139* and *CG17140* (28). This means that the *CG17137* has functional features making them a biologically relevant new member of the Porin/VDAC gene family.

The position of the porin protein in the eukaryotic cell and its involvement in the mitochondrial energy metabolism and in other cellular pathways such as programmed cell death may have raised the need for additional isoforms able to compensate for one another. This could have originated by modern gene duplications in *D. melanogaster*. Today porin isoforms may have similar but not fully coincident functions.

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REFERENCES

1. Schein, S. J., Colombini, M., and Finkelstein, A. (1976) *J. Membr. Biol.* **30,** 99–120
2. Sorgato, M. C., and Moran, O. (1993) *Crit. Rev. Biochem. Mol. Biol.* **18,** 127–171
3. Benz, R. (1994) *Biochim. Biophys. Acta* **1197,** 167–196
4. Rauch, G., and Moran, O. (1994) *Biochem. Biophys. Res. Commun.* **200,** 908–915
5. Guo, X. W., Smith, P. R., Cognon, B., D'Arcangelis, D., Dolginova, E., and Mannella, C. A. (1995) *J. Struct. Biol.* **114,** 41–59
6. Song, J., Midson, C., Blachly-Dyson, E., Forte, M., and Colombini, M. (1998) *J. Biol. Chem.* **273,** 24406–24413
7. Casadio, R., Jacoboni, I., Messina, A., and De Pinto, V. (2002) *FEBS Lett.* **520,** 1–7
8. Schulz, G. E. (2000) *Curr. Opin. Struct. Biol.* **10,** 443–447
9. Rostovtseva, T., and Colombini, M. (1996) *J. Biol. Chem.* **271,** 28006–28008
10. Benz, R., Kottke, M., and Brdiczka, D. (1990) *Biochim. Biophys. Acta* **1022,** 313–318
11. Fiek, C., Benz, R., Roos, N., and Brdzicka, D. (1982) *Biochim. Biophys. Acta* **688,** 429–440
12. Desagher, S., and Martinou, J. C. (2000) *Trends Cell Biol.* **10,** 369–377
13. Tsujimoto, Y., and Shimizu, S. (2002) *Biochimie* (*Paris*) **84,** 187–193
14. Shimizu, S., Narita, M., and Tsujimoto, Y. (1999) *Nature* **399,** 483–487
15. Shimizu, S., Ide, T., Yanagida, T., and Tsujimoto, Y. (2002) *J. Biol. Chem.* **275,** 12321–12325
16. Shimizu, S., Konishi, A., Kodama, T., and Tsujimoto, Y. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 3100–3105
17. Szabo, I., De Pinto, V., and Zoratti, M. (1993) *FEBS Lett.* **330,** 206–210
18. Crompton, M., Virji, S., and Ward, J. M. (1998) *Eur. J. Biochem.* **258,** 729–735
19. Bernardi, P., Petronilli, V., Di Lisa, F., and Forte, M. (2001) *Trends Biochem. Sci.* **26,** 112–117
20. Cheng, E. H. J., Sheiko, T. V., Fisher, J. K., Craigen, W. J., and Korsmeyer, S. J. (2003) *Science* **301,** 513–517
21. Dihanic, M., Suda, K., and Schatz, G. (1987) *EMBO J.* **6,** 723–728
22. Blachly-Dyson, E., Song, J., Wolfgang, W. J., Colombini, M., and Forte, M. (1997) *Mol. Cell. Biol.* **17,** 5727–5738
23. Blachly-Dyson, E., Zambronicz, E. B., Yu, W. H., Adams, V., McCabe, E. R. B., Adelman, J., Colombini, M., and Forte, M. (1993) *J. Biol. Chem.* **268,** 1835–1841
24. Sampson, M. J., Lovell, R. S., and Craigen, W. J. (1997) *J. Biol. Chem.* **272,** 18966–18973
25. Xu, X., Decker, W., Sampson, M. J., Craigen, W. J., and Colombini, M. (1999) *J. Membr. Biol.* **170,** 89–102
26. Messina, A., Neri, M., Perosa, F., Caggese, C., Marino, M., Caizzi, R., and De Pinto, V. (1996) *FEBS Lett.* **384,** 9–13
27. Oliva, M., Messina, A., Ragone, G., Caggese, C., and De Pinto, V. (1998) *FEBS Lett.* **430,** 327–332
28. Oliva, M., De Pinto, V., Barsanti, P., and Caggese, C. (2002) *Mol. Genet. Genomics* **267,** 746–756
29. Popp, B., Court, D. A., Benz, R., Neupert, W., and Lill, R. (1996) *J. Biol. Chem.* **271,** 13593–13599
30. Benz, R., Janko, K., Boos, W., and Lauger, P. (1978) *Biochim. Biophys. Acta* **511,** 305–319
31. Benz, R., Janko, K., and Lauger, P. (1979) *Biochim. Biophys. Acta* **551,** 238–247
32. Jacoboni, I., Martelli, P. L., Fariselli, P., De Pinto, V., and Casadio, R. (2001) *Protein Sci.* **10,** 779–787
33. Martelli, P. L., Fariselli, P., Krogh, A., and Casadio, R. (2002) *Bioinformatics* **18,** S46–S53
34. Eswar, N., John, B., Mirkovic, N., Fiser, A., Ilyin V. A., Pieper, U., Stuart, A. C., Marti-Renom, M. A., Madhusudhan, M. S., Yerkovich, B., and Sali, A. (2003) *Nucleic Acids Res.* **31,** 3375–3380
35. Laskowki, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. (1993) *J. Appl. Crystallogr.* **26,** 283–291
36. De Pinto, V., Benz, R., Caggese, C., and Palmieri, F. (1989) *Biochim. Biophys. Acta* **987,** 1–7
37. Thomas, L., Blachly-Dyson, E., Colombini, M., and Forte, M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 5446–5449
38. Blachly-Dyson, E., and Forte, M. (2001) *IUBMB Life* **52,** 113–118
39. De Pinto, V., Messina, A., Accardi, R., Aiello, R., Guarino, F., Tomasello, M. F., Tommasino, M., Tasco, G., Casadio, R., Benz, R., De Giorgi, F., Ichas, F., Baker, M., and Lawen, A. (2003) *Ital. J. Biochem.* **52,** 17–24
40. Le Mellay, V., Troppmair, J., Benz, R., and Rapp, U. R. (2002) *BMC Cell Biol.* **3,** 14
41. Leterrier, J. F., Rusakov, D. A., Nelson, B. D., and Linden, M. (1994) *Microsc. Res. Tech.* **27,** 233–261
42. Xu, X., Forbes, J. G., and Colombini, M. (2001) *J. Membr. Biol.* **180,** 73–81
43. Komarov, A. G., Graham, B. H., Craigen, W. J., and Colombini, M. (2004) *Biophys. J.* **86,** 152–162
44. Popp, B., Schmid, A., and Benz, R. (1995) *Biochemistry* **34,** 3352–3361
45. Forte, M., Guy, H. R., and Mannella, C. A. (1987) *J. Bioenerg. Biomembr.* **19,** 341–350
46. Blachly-Dyson, E., Peng, S. Z., Colombini, M., and Forte, M. (1990) *Science* **247,** 1233–1236
47. Zizi, M., Thomas, L., Blachly-Dyson, E., Forte, M., and Colombini, M. (1995) *J. Membr. Biol.* **144,** 121–129