Cloning and chromosomal localization of a cDNA encoding a mitochondrial porin from *Drosophila melanogaster*

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Abstract **We have raised polyclonal antibodies against purified the *Drosphila melanogaster* mitochondrial porin. They showed high titre and specificity and were thus used as a tool for screening an expression library. The isolated clone 1T1 showed 74% sequence identity in the last 19 residues at the C-terminus of human porin. A subclone of 1T1, containing the porin-like sequence, was thus used as a probe for re-screening a cDNA library and several positive clones were plaque-purified. We present here the sequence of a 1363 bp cDNA encoding a protein of 279 amino acids. Its identity with porin was also confirmed by N-terminal Edman degradation of the purified protein. The *D. melanogaster* porin shows an overall 51.8% identity with human porin isoform 1 (porin 31HL or HVDAC1) and an overall 55.7% identity with human porin isoform 2 (HVDAC2). Hydrophobicity plots and secondary structure predictions showed a very high similarity with data obtained from known porin sequences. The *D. melanogaster* porin cDNA was used as a probe for in situ hybridization to polytenic salivar gland chromosomes. It hybridizes with different intensities in two sites, in chromosome 2L, at region 31E and in chromosome 3L at region 79D. Thus, also in *Drosophila melanogaster* porin polypeptide(s) belong(s) to a multigene family.**

Key words: Porin pore; *Drosophila melanogaster*; cDNA; Sequence; Chromosomal localization; Mitochondria

1. Introduction

Gram-negative bacteria and mitochondria share in many respects a similar organization. Both are surrounded by two membranes and mitochondria have an autonomous genetic system closer to the bacterial than to the nuclear one. In their outer membranes channels are present, called porins, which show a similar basic function, i.e. they are sieving gates for polar metabolites [1,2]. Nevertheless, bacterial and mitochondrial porins have functionally relevant differences, like different ionic selectivity and the sensitivity to voltage gradients across the membrane, which was detected only in mitochondrial porins (for reviews see [3,4]). From the structural point of view, bacterial porins were crystallized and the most refined analysis showed a very typical organization of the pore which is composed by a number of amphipathic β-sheet barrels [5,6]. There are indications that mitochondrial porins also contain this very unique secondary structure [7,8].

Furthermore, like bacteria, which have in their genome several genes for porin with general or specific functions (i.e. OmpC, OmpF, LamB, PhoE in strains of *E.coli*), in eukaryotics a similar picture is also emerging from the most recent studies. At least four different genes [9], two of which have shown to be transcribed [10], were identified in humans: interestingly the protein expressed by these two mRNAs in VDAC-null yeast showed a striking difference in the hexokinase-binding property, which was conserved only by one of them [10]. Also in plants, families or groups of porins were identified [11,13].

The role of this 'porin redundancy' is not yet clear. The redundancy could witness the role of porin(s), a crucial linking point joining the mitochondrial with the rest-of-the-cell metabolism. Since the permeability of the outer mitochondrial membrane is essential, the existence of one or more 'rescue' channel-proteins must be hypothesized. This possibility is strengthened by the recovery of porin yeast-null mutants characterized by an electrophysiologically novel pore-forming activity [14–16]. On the other hand, a differential tissue expression could be invoked, as shown in wheat [13]. The first discovered porin-deficient human patient shows a lack of porin in muscle but its presence in fibroblasts [17,18]: this may be due to damage to the tissue-specification genetic machinery. At the end, the different subcellular localization of porin isoforms may be involved, as indicated in plants, where both amyloplast and mitochondria have been shown to contain porins with very similar functional and structural features [11].

To raise our chances of understanding the multigene family which underlies the mitochondrial porin(s) expression, we thus decided to clone porin(s) from *Drosophila melanogaster*, a most suitable eukaryotic organism, from both the genetic and biochemical point of view. In this work we present our first step in this direction.

2. Material and methods

2.1. Porin purification and immunological techniques

Porin was purified from adult flies (*Oregon-R*) mitochondria by means of a previously described procedure [19,20]. The purified protein was run on SDS-PAGE and repurified by cutting the gel slices corresponding to the 31 kDa polypeptide. Porin was electroeluted from the gel and this material was emulsified with Freund adjuvant and injected intraperitoneally in three BALB/C mice. The serum titre was checked after three and five boosters and serum aliquots collected 1 week after the last booster. ELISA assay and Western blot on the sera were performed as described [21,22].

2.2. cDNA library screening

A cDNA library from heads of adult flies in λgt11 (kindly provided by Mike Forte, Oregon) was used for both immunological and colony hybridization screenings. Immunological screening was performed with the usual procedures. Filters were incubated with the primary

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antiserum at a 1:500 dilution. Staining was with 3,3′-diaminobenzidine (DAB).

Colony hybridization was performed upon the same cDNA library with the ^{32}P-labeled 1T1 *Eco*RI/*Cla*I subclone in high stringency conditions.

2.3. DNA sequencing and computer analysis

DNA sequencing of double-stranded plasmid DNA was accomplished using the dideoxy chain termination method with Sequenase ver. 2.0 according to the manufacturer's specifications and using synthetic oligonucleotides as primers (Genset, Paris, France). The nucleic acid and the deduced amino acid sequence of the *D. melanogaster* porin were analyzed using the GCG package (University of Wisconsin).

2.4. In situ hybridization to polytene chromosomes

In situ hybridization to polytene chromosomes was performed essentially as described in [23] except that non-radioactive biotinylated probes were used. Chromosomes were stained with DAPI and hybridization sites were detected using fluorescein-isothiocyanate (FITC)-conjugated avidin. Digital images were obtained using a computer-controlled Zeiss Axioscope epifluorescence microscope equipped with a cooled charge-coupled device camera (CCD, Photometrics). FITC and DAPI fluorescence, detected by specific filter combinations, was recorded separately as gray scale images and then merged for the final image.

3. Results

Porin was purified from adult flies [20] and used as an antigen to raise antibodies in mice. The antiserum was highly reactive against *Drosophila* porin, as assessed by ELISA assay (Fig. 1) and Western blots (not shown). The antibodies were thus used for screening a λgt11 *D. melanogaster* cDNA library. A single positive (called 1T1) was collected among 250 000 clones screened. It was subcloned in pUC 19 and the whole sequence of the insert determined. The insert cross-hybridized with human mitochondrial porin cDNA.

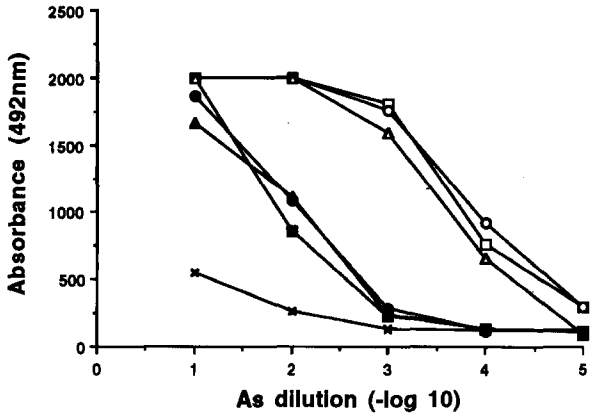

Fig. 1. ELISA assay for three polyclonal anti-*D. melanogaster* porin antisera. 50 μl of various dilutions of sera obtained from three BALB/C mice immunized with purified *D. melanogaster* porin were added to 96-well microtiter plates previously coated with purified bovine heart porin (BHP; closed symbols) and *D. melanogaster* porin (DmP; open symbols). Following a 4 h incubation at 25°C and washings of the plates, bound Ab were detected by the sequential addition of affinity-purified peroxidase conjugated xeno-Abs[F(ab′)$_2$ fragments] to the Fc portion of mouse IgG (Jackson Immunoresearch Lab., Avondale, PA) and freshly prepared OPD-bound substrate solution. Background binding was determined by replacing test samples with RPMI 1640 containing 10% fetal calf serum. Binding of pre-immune BALB/C mouse sera (crosses) was considered as control.

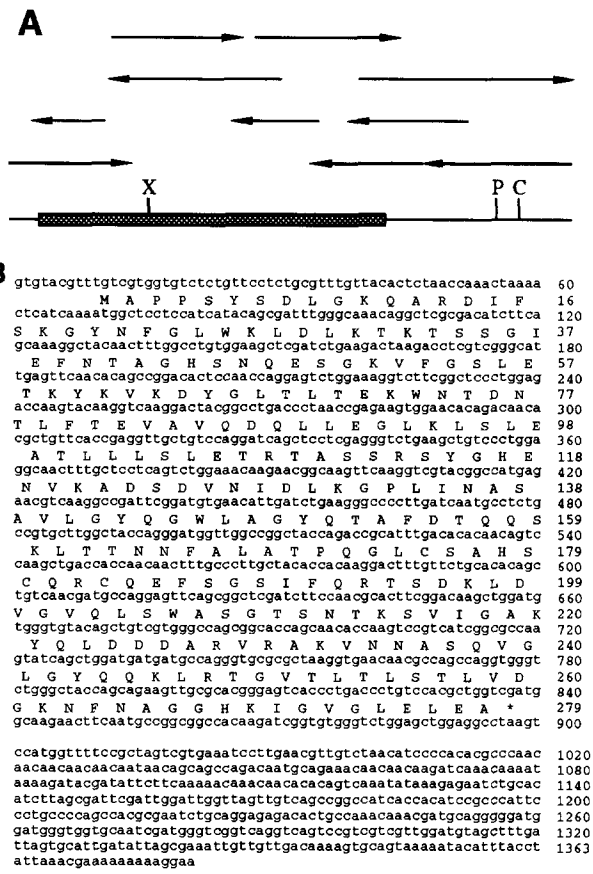

Fig. 2. (A) Structure of *D. melanogaster* porin cDNA. The putative coding region is indicated by a box. Restriction sites are marked for the restriction enzymes: P, *Pst*I; C, *Cla*I; X, *Xho*I. Arrows denote the direction and extent of sequencing for the clone 7T21. (B) Nucleotide and deduced amino acid sequence of *D. melanogaster* porin cDNA. The corresponding amino acid sequence is indicated above the nucleotide sequence. The stop codon is indicated by an asterisk.

The sequence was chimeric: it showed the presence of the 3′-terminal part of an unknown message curiously fused with bp 4519–4954 of the sequence of *D. melanogaster* CitOx subunit III, which is mitochondrially encoded, until a native *Eco*RI site (4949–4954) [24,25]. The cDNA sequence fused to the CitOx subunit was an open reading frame for the C-terminus of a protein. The contamination of nuclear cDNA by mtDNA at the beginning or at the end of untranslated regions was reported [26]: it is not clear whether it is naturally occurring or just an artifact [26]. Comparison in the database showed that the unknown open reading frame was highly homologous to the C-terminus of the human porins 31HL and HVDAC2.

On account of a *Cla*I site located at the end of the unknown part of 1T1, it was possible to subclone an *Eco*RI/*Cla*I 382 bp fragment which excluded the CitOx sequence. The 1T1 *Eco*RI/*Cla*I subclone was thus used as a probe to isolate larger cDNA clones. Several messages were collected. The insert of a long cDNA (clone 7T21) was sequenced completely on both strands using specific oligonucleotide primers based on the newly acquired sequence. The sequence of the 7T21 3′-region was identical to the 1T1 *Eco*RI/*Cla*I subclone. Its complete nucleotide sequence is shown in Fig. 2 (accession no. X-92408). The coding region contains 840 nucleotides, including the starting ATG. No in-frame stop codon precedes the pre-

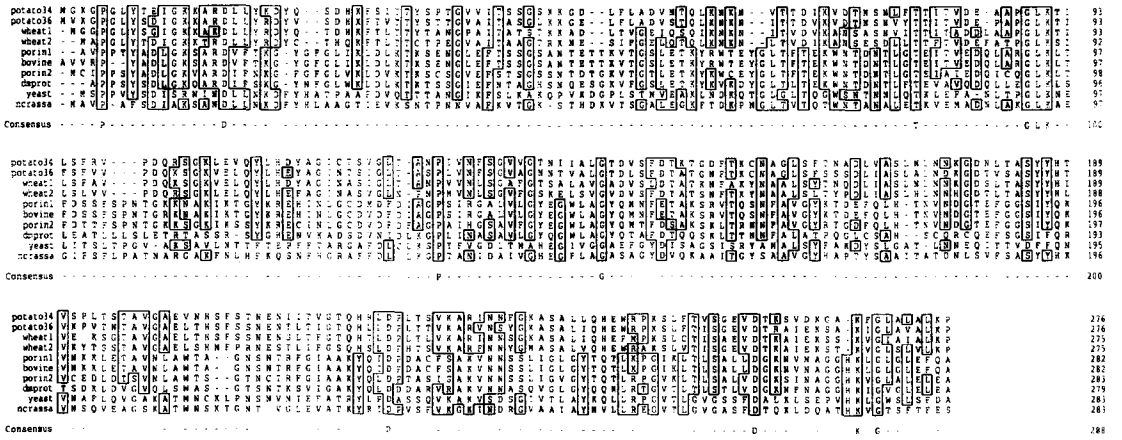

Fig. 3. Comparison of the amino acid sequences of human1 (porin 31HL) [27], human2 (HVDAC2) [10], bovine [28], *D. melanogaster* (dmprot), yeast [29], *N. crassa* [7], two isoforms from potato (potato34 and potato36) [12] and two isoforms from wheat (wheat1 and wheat2) [13] porins. Identical amino acid are boxed. The eleventh line shows the identical residues in the 10 sequences as a consensus.

dicted open reading frame. The 5′-non coding region is relatively short (77 bp) while the 3′-non coding region extends for 450 bp, including a short poly(A) tail.

The identity of this protein as *D. melanogaster* mitochondrial porin was further confirmed by Edman degradation of the N-terminal sequence of the purified porin polypeptide. The N-terminus was not blocked and showed the sequence: APPSYSDLG. This indicates that the starting Met is not translated in the mature protein.

The open reading frame of *D. melanogaster* cDNA corresponds to a protein consisting of 279 amino acids. Its deduced molecular weight was 30 189 Da, in good agreement with that estimated by SDS-polyacrylamide gel electrophoresis (31 kDa [20]). Its amino acid content, as usual in porins, is not specially hydrophobic. The only missing residue is Met.

Sequence comparison in the database gave the following identity score in comparison with other known porins: HVDAC2 [10] (55.7%), porin 31HL [27,10] (51.8%), bovine [28] (52%), *N. crassa* [7] (30.3%), wheat [13] (28.6%), *Zea mays* [11] (26.9%), 34 kDa potato [12] (25.8%), *S. cerevisiae* [29] (24.6%), 36 kDa potato [12] (23.8%) and *Dictyostelium discoides* [30] (23.4%). In Fig. 3 a comparison of most of the known mitochondrial porins is shown. The consensus sequence, which shows the perfect identity in the sequences reported, is limited to only 12 amino acids. The most conserved regions are the N-terminal and C-terminal. The identical residues are shed in the sequence and are mostly charged amino acids (D,K) or residues with some structural influence (G,P). The most striking identity is the peptide GLK (about 92–94) whose meaning is not known, but which is conserved in all the porin sequences known with the exception of the pea amyloplast protein, which has GVK [11].

Fig. 4 demonstrates the similarity in the secondary structure among *D. melanogaster* porin and other known porins. The analysis with a method proposed by Vogel [31] revealed the presence of rather short stretches of alternating hydrophilic and hydrophobic amino acids, a typical output for amphipathic β-strands [7].

When used as a probe for in situ hybridization upon polytenic salivary gland chromosomes, the porin cDNA showed signals on two chromosomes (Fig. 5): chromosome 2L, at the region 31E and chromosome 3L at region 79D. The two signals were of different intensity: the signal at 79D was stronger than that at 31E. This result indicates that there are at least two loci containing sequences of similar homology to the reported cDNA. Experiments are in progress to clarify if one or both chromosomal loci are expressing genes for porin cDNA.

4. Discussion

In gram-negative bacteria one or two porins (i.e. OmpF and OmpC in *E. coli* K-12 [32]) are present in the outer membrane under normal growth conditions. During metabolic stress or in the presence of an unbalanced nutrient condition, other porins forming general diffusion pores (i.e. PhoE porin [33]) or specific pores with small selectivity (i.e. protein P [34]) are inducible. Also, in eukaryotics a similar picture is emerging from the most recent studies. The first evidence came from biochemical investigations. In yeast the disruption of the porin gene resulted in viable yeast mutants [14–16]. They show efficient adenine nucleotide exchange across the outer membrane in vivo [15] and a defined single channel conductance upon reconstitution of detergent-solubilized mitochondrial outer membrane in planar lipid bilayers [16]. Since no new protein was induced in the electrophoretic pattern of mutant yeast mitochondrial outer membrane, it was suggested that the 'second' or 'recovery' porin is also present in wild-type mitochondria [35].

In humans the presence of porin in the cytoplasmic membrane of cultured B-lymphocyte cells was shown by indirect immunofluorescence staining with specific anti-peptide antibodies [36]. Its physiological role is still unknown. Furthermore, reports showed the presence of at least four genes for porin(s) in the genome. The four genes mapped at different chromosomes [9]. Three of them, namely porin 31HL or HVDAC1, HVDAC3 and HVDAC4, appear strictly correlated since they show around 95% identity even in the 3′-untranslated regions [9]. On the other hand, HVDAC2 diverges considerably in the 3′-untranslated region and has 11 more amino acids at the N-terminus [10]. Northern blot analysis in different tissues indicated that porin 31HL and HVDAC2 are expressed in almost all the tissues [10]. It is still unknown whether HVDAC3 and 4 are pseudogenes.

Additional information about expression of human porin

was reported by Ha et al. [37]. They obtained a cDNA containing an open reading frame which is essentially the same as HVDAC2. It is identical between nucleotide 324 (corresponding to 94 in HVDAC2) and 1464 (1235 in HVDAC2) with three exceptions. The most noticeable is the lack of a G (position 921 in HVDAC2) which shifts the HVDAC2 stop codon and changes the C-terminal by adding 38 more amino acids. In addition, the cDNA isolated by Ha et al. shows a further extension at the 5′-end with a new potential initiation codon. In conclusion, two mRNAs and two proteins, produced most likely from the same gene, were claimed to be expressed in various human cell lines [37].

Another piece of evidence that a differential expression of human genes for porin exists comes from the recent finding of a human patient lacking porin (porin 31HL) in his muscle [17]. The child showed severe problems but survived. This means that the mitochondrial activity was allowed by a 'second' or by a 'recovery' porin. Then, in fibroblasts from the same patient, the same porin polypeptide missing in muscle could be detected [18]. As a consequence a tissue-regulated expression of porin gene(s) must be hypothesized.

In plants non-green plastidial porins were recently cloned [11]. Their sequence and secondary structure are remarkably similar to those of their cognate plant mitochondrial porins. This raises the exciting possibility that similar proteins with totally different structures for organelle-specific importing mechanisms developed from a common ancestor [11]. In wheat Elkeles et al. [13] reported the sequence of three cDNAs for porin: they show 65% similarity of their sequences and map at three different chromosomes. Interestingly different expression levels were detected both at a tissue level and in the embryo's development [13]. The three porin transcripts could perform different functions as could be suspected by their relative abundances.

In this work we have isolated and sequenced a cDNA corresponding to a mRNA for a *D. melanogaster* porin. The identity of this cDNA as a mitochondrial porin was confirmed both by its isolation with a specific anti-porin antiserum and by Edman degradation of the N-terminal sequence of the purified antigenic porin polypeptide. The complete primary structure of this insect porin cDNA contains an open reading frame of 279 amino acids and is highly homologous to human porins 31HL and HVDAC2.

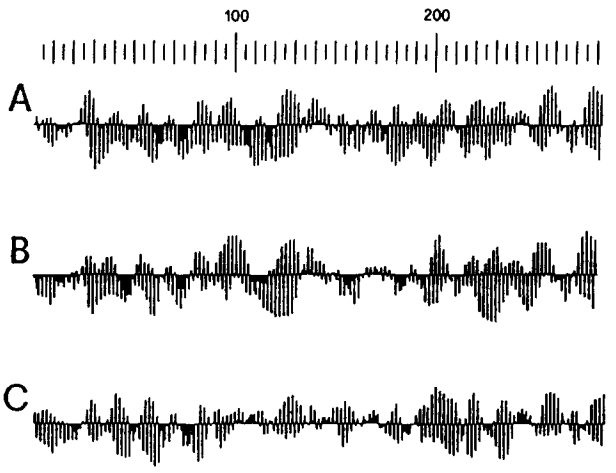

Fig. 4. Sided-hydropathy profiles of mitochondrial porins from (A) human (porin 31HL), (B) *D. melanogaster* and (C) yeast. The hydropathy values for sided β-strands were obtained by summing up the hydropathy indices, $H(n)$, of the respective amino acid residues (from [39], using the following weights: $0.5 \times H(n-4) + H(n-2) + H(n) + H(n+2) + 0.5H(n+4)$. The sum was calculated for each residue n and plotted as a function of the number of residues. Possible amphipathic β-sheets can be detected in the plot by an alteration between hydrophilic and hydrophobic values every second residue [31].

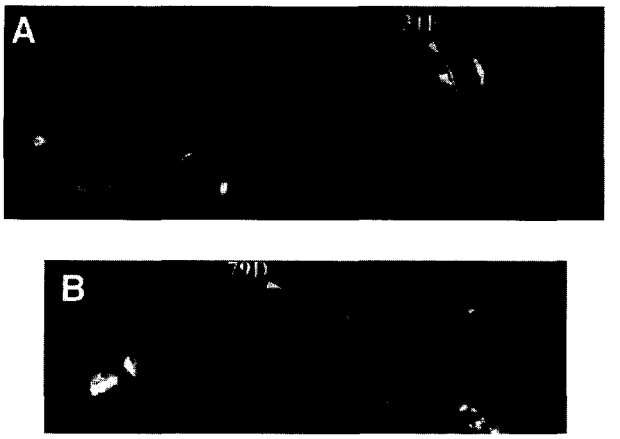

Fig. 5. Cytological localization of porin cDNA (clone 7T21) to polytene chromosomes of *Oregon-R D. melanogaster*. Squashes and hybridization with the porin cDNA 7T21 as a probe were performed as in [23]. In A a particular of chromosome 2L is reported; in B a particular of chromosome 3L with the chromocenter is shown.

Not unexpectedly, when used as a probe for in situ hybridization to polytenic salivary gland chromosomes, this cDNA hybridized at two different loci. The intensity of the staining in the hybridization experiments was different for the two loci: stronger at the region 79D than at 31E. This should in turn mean that the two genomic sequences are somehow different. It is thus likely that also in the insect genome a multigene family underlies the expression of porin(s).

The use of different genes for different functions or the alternate expression of the same gene(s) could account for the number of putative functions attributed to porin(s) by biochemical experiments. We are indeed working towards the isolation and sequencing of *D. melanogaster* genes to explain at a molecular level their differential expression.

D. melanogaster is a eukaryotic system well known for its similarity to man and for its convenience and ease of study [38], following the principle that 'if you've seen one species, you've seen them all'. Also on this occasion the very high degree of homology with human indicates that this approach could be a useful key to clarify the genetic system which underlies the porin(s).

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