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Identification of the *Drosophila melanogaster* Mitochondrial Citrate Carrier: Bacterial Expression, Reconstitution, Functional Characterization and Developmental Distribution

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The mitochondrial carriers are a family of transport proteins that shuttle metabolites, nucleotides and cofactors across the inner mitochondrial membrane. The genome of *Drosophila melanogaster* encodes at least 46 members of this family. Only four of them have been characterized: the two isoforms of the ADP/ATP translocase, the brain uncoupling protein and the carnitine/acylcarnitine carriers. The transport functions of the remainders cannot be assessed with certainty. One of them, the product of the gene CG6782, shows a fairly close sequence homology to the known sequence of the rat mitochondrial citrate carrier. In this article the fruit fly protein coding by the CG6782 gene has been functionally characterized by over-expression in *Escherichia coli* and reconstitution into liposomes. It shows to have similar transport properties of the eukaryotic mitochondrial citrate carriers previously biochemically characterized. This indicates that in addition to the protein sequence conservation, insect and mammalian citrate carriers are also significantly related at the functional level suggesting that Drosophila may be used as model organism for the study of mitochondrial solute transporter. The *Dm*CIC expression pattern throughout development was also investigated; the transcripts were detected at equal levels in all stages analysed.

Key words: citrate carrier, *Drosophila melanogaster*, metabolite transporter, mitochondria, transport.

Abbreviations: BAT, Bathophenanthroline; BTA, benzenetricarboxylic acid; *Dm*CIC, *Drosophila melanogaster* citrate carrier; PIPES, 1,4-piperazinediethanesulfonic acid; PLP, Pyridoxal 5′-phosphate; RT-PCR, reverse transcription polymerase chain reaction.

The tricarboxylate (or citrate) carrier (CIC) is a transport protein of the inner mitochondrial membrane, which belongs to the mitochondrial carrier family [for review see (*1*)]. The CIC catalyses the transport of citrate in exchange for a tricarboxylate, a dicarboxylate (L-malate) or phosphoenolpyruvate (*2–4*). This carrier protein plays a central role in intermediary metabolism because it supplies the cytosol with acetyl units (deriving from the transported citrate) necessary for the de novo fatty acid and cholesterol biosyntheses (*5*). The CIC has been kinetically characterized in eukaryotes (*6–8*) and its primary structure has been determined (*7*, *9–11*). However, the functional characterization of this particular mitochondrial solute carrier in model organisms is still missing.

In this study, we have examined the proteins encoded on the genome of *Drosophila melanogaster* for members of the mitochondrial carrier family, and we have identified 46 members (data not shown). One of them, encoded by gene CG6782 (http://www.flybase.org), displays a high identity (*i.e.* 62%) to the rat citrate carrier protein. We therefore have expressed and characterized the protein encoded by this sequence, afterwards named *Dm*CIC. In the present article, we report (i) the expression and purification of large quantities of the fruit fly *Dm*CIC, (ii) the functional characterization of the over-expressed *Dm*CIC following its incorporation into liposomes and (iii) a expression profile of *Dm*CIC by RT-PCR analysis in different developmental stages. To our knowledge, this report presents the first information on the molecular properties of the fruit fly *Dm*CIC and a definitive identification of its gene.

MATERIALS AND METHODS

Computer Search for DmCIC—The proteins annotated in the FlyBase were compared with the rat sequence of the mitochondrial citrate carrier (P32089) with the aid of the program blastp.

Construction of the Expression Plasmid Coding for Mature DmCIC—Total RNA was extracted from Oregon

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R adult flies using RNeasy Mini Kit (Quiagen) and reverse transcribed as described in (*12*). The mature coding region for the *Dm*CIC was amplified from first strand cDNA (100 ng) by polymerase chain reaction with nucleotides 164–180 and 1011–1036 of the fruit fly transcript CG6782-RA. The product was cloned into the NdeI-HindIII sites of the expression vector pET-21b that it had been previously modified by cloning into HindIII-XhoI sites a cDNA sequence coding for a V5 epitope followed by six histidines (pET-21b/V5/His). Transformants of *Escherichia coli* TG1 cells were selected and screened. The absence of the stop codon in the reverse primer sequence led to the expression of the *Dm*CIC with carboxy-terminal V5- and His6-epitope tag. The *Dm*CIC protein was overexpressed in *E. coli* in BL21 (DE3). Inclusion bodies were isolated and *Dm*CIC was purified by centrifugation and Ni^{+}-NTA-agarose affinity chromatography as described (*13*).

Reconstitution into Liposomes and Transport Assays—The recombinant protein in sarkosyl was reconstituted into liposomes in the presence of substrates as described (*14*). External substrate was removed on Sephadex G-75. Transport at 25°C was started by adding [^{14}C]citrate (from Amersham) to proteoliposomes and terminated by addition of 20 mM 1,2,3-BTA. In controls, inhibitor was added with the labelled substrate. All transport measurements were carried out at the same internal and external pH values (PIPES 10 mM, pH 7.0). Finally, the external substrate was removed, and the radioactivity in the liposomes was measured (*14*).

RNA Isolation and Semiquantitative RT-PCR—Total RNA was isolated from wild-type Oregon R embryos, larvae, pupae and adult flies using RNeasy Mini Kit (Qiagen). Fifty nanograms of RNA were reverse transcribed and amplified using Access RT-PCR System kit (Promega). The *twinstar (tsr)* gene was used as an internal control. The sense and antisense gene-specific primers were as follows: *Dm*CIC, sense CTGTCCCCATCGCCACTTCA, anti sense CTATAGCCACTTACCCATTGC; tsr, sense TTGTTCGTGA AA, antisense ATACGTGTTTCC. The PCR products were analysed by 1% agarose gel electrophoresis. Band intensities were quantified using Quantity One 1-D Analysis Software (Biorad, Hercules, CA).

Other Methods—Proteins were analysed by SDS–PAGE and stained with Coomassie Blue dye or transferred to nitrocellulose membranes. The amount of pure *Dm*CIC was estimated by laser densitometry of stained samples using carbonic anhydrase as a protein standard. The amount of protein incorporated into liposomes was measured as described previously (*12*). Western blotting was carried out with mouse anti-V5 monoclonal antibody as described previously (*15*).

RESULTS AND DISCUSSION

Bacterial Expression of Recombinant DmCIC—By screening *Drosophila melanogaster* non-redundant databases with the rat CIC (P32089), a gene CG6782 encoding for the putative fruit fly citrate carrier, *Dm*CIC, was found.

The multiple clustalW alignment (data not shown) of the the *Dm*CIC with mature citrate carrier of rat and silver eel, which N-terminal sequences were determined by direct sequence of purified native proteins (*9*, *11*), shows that the mature *Dm*CIC starts with residues ADSG, indicating that fruit fly *Dm*CIC is processed between residues 26 and 27.

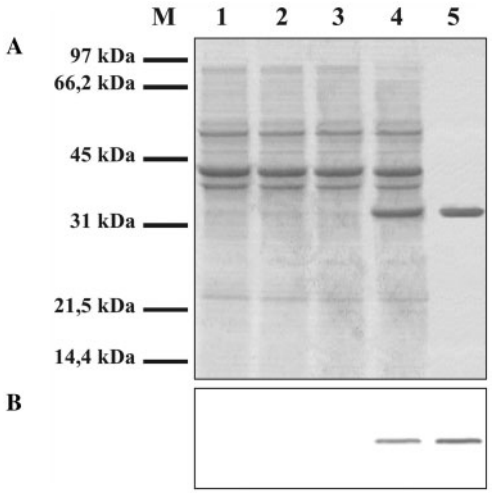

Fig. 1. **Expression in *E. coli* and purification of the *Dm*CIC.** Proteins were separated by SDS–PAGE and stained with Coomassie-blue dye (A) or transferred to nitrocellulose and immunodecorated with mouse anti-V5 monoclonal antibody (B). Lanes M, markers (Phosphorylase b, Serum albumin, Ovalbumin, Carbonic anhydrase, Trypsin inhibitor and Lysozyme); lanes 1–4, *E. coli* BL21(DE3) containing the expression vector, without (Panels A and B, lanes 1 and 3) and with the coding sequence for *Dm*CIC (Panels A and B, lanes 2 and 4). Samples were taken at the time of induction (lanes 1 and 2) and 4 h later (lanes 3 and 4). The same number of bacteria was analysed in each sample. Lane 5, purified *Dm*CIC (5 micrograms) originating from bacteria shown in lane 4.

The mature *Dm*CIC was overexpressed in *E. coli* BL21(DE3) at high levels (see Fig. 1A, lane 4). The protein was not detected in bacteria harvested immediately before induction of expression (Fig. 1A, lanes 1 and 2), nor in cells harvested after induction and transformed with empty vector (Fig. 1A, lane 3). The purified protein showed a single band when analysed by SDS–PAGE (Fig. 1A, lane 5), with an apparent molecular mass of about 33 kDa. Its identity was confirmed by western blot analysis using mouse anti-V5 monoclonal antibody (Fig. 1B, lanes 4 and 5).

Functional Characterization of DmCIC—The *Dm*CIC recombinant protein was reconstituted into liposomes and the uptake of 1 mM [^{14}C]citrate was measured either as uniport (in the absence of internal citrate) or as exchange (in the presence of 20 mM citrate). The uptake of citrate by exchange followed a first order kinetics (rate constant 0.147/min; initial rate, 9.25 μmol/min/mg of protein) with isotopic equilibrium being approached exponentially (*14*). In contrast, no [^{14}C]citrate uptake was observed without internal substrate, demonstrating that *Dm*CIC does not catalyse unidirectional transport (uniport) but only an exchange of substrates (data not shown). The exchange reaction was completely inhibited by 1,2,3-BTA. If the solubilized protein was boiled before the incorporation into liposomes no uptake of labelled external substrate into proteoliposomes was observed.

Similarly, no citrate/citrate exchange was detected when sarkosyl-solubilized material from bacterial cells, either lacking the expression vector for *Dm*CIC or harvested immediately before induction of expression, was reconstituted into liposomes. Furthermore, the proteoliposomes did not catalyse homoexchange activities for phosphate, glutamate, aspartate, ornithine, ADP and ATP (internal concentration, 20 mM; external concentration, 1 mM).

The substrate specificity of recombinant *Dm*CIC was extensively investigated by measuring the uptake of [^{14}C]citrate into proteoliposomes preloaded with various substrates. As shown in Fig. 2, the highest activities were observed in the presence of internal *cis*-aconitate, threo-isocitrate, phosph*enol*pyruvate and L-malate. Some activity was also observed with succinate. Furthermore, no significant exchange activity was found using *trans*-aconitate, or substrates of other mitochondrial carriers, like phosphate, 2-oxoglutarate, glutamate and ATP. These results indicate clearly that radioactive citrate is transported into proteoliposomes containing *Dm*CIC, only in exchange for an internal counter-substrate.

The sensitivity of the reconstituted citrate/citrate exchange to externally added inhibitors was investigated (Fig. 3). The 1,2,3-BTA was more effective than its structural isomers 1,2,4-BTA and 1,3,5-BTA. The activity of the recombinant protein was markedly inhibited by PLP and BAT (known inhibitors of several mitochondrial carriers) as well as by thiol reagents (mersalyl and mercuric chloride), whereas a much higher amount of *N*-ethylmaleimide was required for a partial inhibition. The different inhibitory potency of these SH reagents can be explained, at least in part, by taking into account the microenvironment surrounding the reactive cysteine(s). Therefore, the binding of such reagents with a specific SH-group could be differently affected by amino acid residues surrounding it. *Dm*CIC was inhibited slightly by impermeable dicarboxylate analogues butylmalonate and phenylsuccinate, which are known to be powerful inhibitors of the dicarboxylate and oxoglutarate carriers, respectively (*2*).

The transport characteristics of the recombinant *Dm*CIC and the effects of inhibitors on transport are in agreement with those determinated for rat and silver eel mitochondrial CIC (*3*, *8*, *16*).

The kinetic constants of the recombinant purified CIC were determined by measuring the initial transport rate at various external [^{14}C]citrate concentrations, in the presence of a constant saturating internal concentration (20 mM) of citrate. The K_m and V_{max} values (measured at 25°C) were 132 ± 0.8 μM and 11.75 ± 0.133 μmol/min/mg protein, respectively (four experiments). The activity was calculated by taking into account the amount of CIC recovered in the proteoliposomes after reconstitution. The half-saturation constant of citrate of *Dm*CIC is similar than that determined for the tricarboxylate carrier of rat liver (*3*, *16*) while is higher than those determined for yeast and silver eel CIC (*7*, *8*).

From its high sequence identity with rat CIC, transport properties and kinetic characteristics there is no doubt that the fruit fly *Dm*CIC is the mitochondrial citrate carrier protein and that the *Drosophila melanogaster* could be used as model organism for the studies of mitochondrial solute transporters.

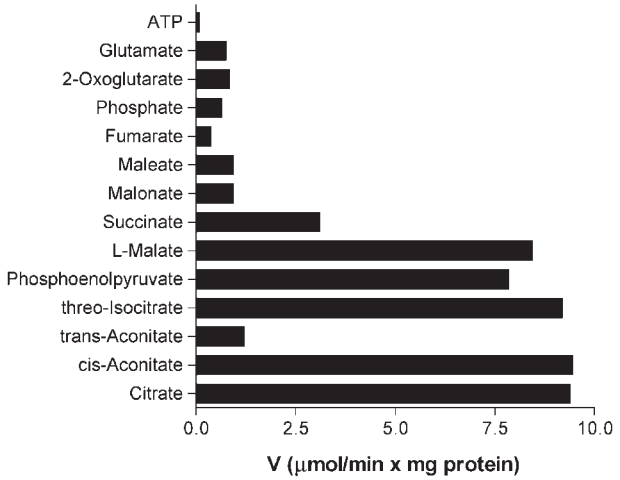

Fig. 2. **Dependence of *Dm*CIC activity on internal substrate.** Proteoliposomes were preloaded internally with various substrates (20 mM). Transport was started by adding 150 μM [^{14}C]citrate to proteoliposomes reconstituted with *Dm*CIC, and stopped after 1 min. Similar results were obtained in at least three independent experiments.

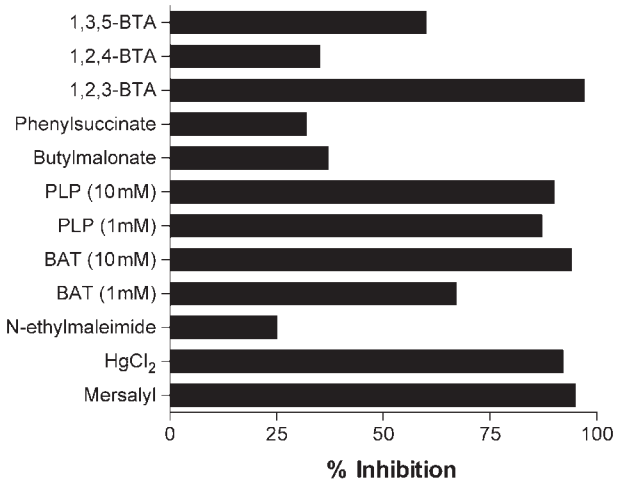

Fig. 3. **Effect of inhibitors on the [^{14}C]citrate/citrate exchange by *Dm*CIC.** Transport was started by adding [^{14}C]citrate (final concentration 150 μM) to proteoliposomes containing 20 mM citrate. Thiol reagents were added 2 min before the labelled substrate, the other inhibitors together with labelled substrate. The final concentrations of inhibitors were 0.1 mM (mersalyl, *p*-hydroxymercuribenzoate and $HgCl_2$), 10 mM (BAT and PLP), 1 mM (BAT, PLP and *N*-ethylmaleimide), 2 mM (1,2,3-BTA; 1,2,4- BTA; 1,3,5- BTA). The control value for uninhibited citrate uptake was 9.455 mmol/min/mg protein. The data represent means of at least four independent experiments.

RT-PCR—To determine the expression levels of *Dm*CIC, we performed a RT-PCR analysis on mRNAs from wild-type embryos, larvae, pupae and adults (Fig. 4). This analysis revealed that *Dm*CIC appears to be equally transcribed at each stage suggesting that its expression is required during the fruit fly development.

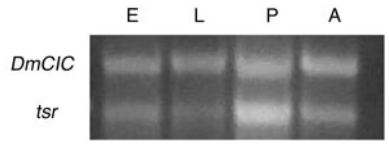

Fig. 4. **Expression of *Dm*CIC during development.** RT-PCR for *Dm*CIC and *tsr* (internal control) was carried out on RNAs obtained from Oregon R embryos (E), larvae (L), pupae (P) and adults (A). Quantification of 1.3 kb *Dm*CIC products relative to *tsr* in each stage has revealed that the levels of *Dm*CIC transcripts do not significantly differ during development.

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