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Abundant expression and purification of biologically active mitochondrial citrate carrier in baculovirus-infected insect cells

Marianna Madeo · Chiara Carrisi · Domenico Iacopetta · Loredana Capobianco · Anna Rita Cappello · Cecilia Bucci · Ferdinando Palmieri · Giancarlo Mazzeo · Anna Montalto · Vincenza Dolce

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Abstract Heterologous expression of recombinant proteins is an essential technology for protein characterization. A major obstacle to investigating the biochemical properties of membrane proteins is the difficulty in obtaining sufficient amounts of functional protein. Here we report the successful expression of the tricarboxylate (or citrate) carrier (CIC) of eel (*Anguilla anguilla*) from *Spodoptera frugiperda* (Sf9) cells using the baculovirus expression system. The recombinant CIC was purified by affinity chromatography on Ni^{2+}-NTA agarose; the yield of the purified active protein was 0.4–0.5 mg/l of culture. The transport characteristics of the recombinant CIC and the effects of inhibitors on transport are similar to those determined for eel liver mitochondrial CIC. Because the CIC is one member of an extensive family of mitochondrial transport proteins, it is likely that the procedure used in this study to express and purify this carrier can be successfully applied to other mitochondrial transport proteins, thus providing sufficient protein for functional characterization.

Keywords Mitochondria · Citrate carrier · Membrane protein · Detergents · V5/His-tag · Baculovirus expression

Abbreviations

BAT	bathophenanthroline
BTA	benzenetricarboxylate
CIC	citrate carrier
DDM	n-dodecyl β -D-maltopyranoside
OG	n-octyl β-D-glucopyranoside
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PLP	pyridoxal-5'-phosphate
PMSF	phenylmethylsulfonyl fluoride
SDS-PAGE	polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate
TX-100	Triton X-100
TX-114	Triton X-114

Introduction

The tricarboxylate (or citrate) carrier (CIC) is a transport protein of the inner mitochondrial membrane, which belongs to the mitochondrial carrier family (Palmieri 2004; Palmieri 2008). The CIC catalyzes the transport of citrate in exchange for a tricarboxylate, a dicarboxylate (L-malate) or phosphoenolpyruvate (Palmieri et al. 1972). This carrier plays a central role in intermediary metabo-

Marianna Madeo and Chiara Carrisi contributed equally to this work.

M. Madeo · D. Iacopetta · A. R. Cappello · G. Mazzeo · A. Montalto · V. Dolce (✉)
Department of Pharmaco-Biology, University of Calabria, 87036 Cosenza, Italy
e-mail: vdolce@unical.it

C. Carrisi · L. Capobianco (✉) · C. Bucci
Department of Biological and Environmental Sciences and Technologies, University of Salento, 73100 Lecce, Italy
e-mail: loredana.capobianco@unile.it

F. Palmieri
Department of Pharmaco-Biology, University of Bari, Via E. Orabona 4, 70125 Bari, Italy

F. Palmieri
CNR Institute of Biomembranes and Bioenergetics, Bari, Italy

lism because it supplies the cytosol with acetyl units (derived from transported citrate) necessary for de novo fatty acid and cholesterol biosynthesis (Meijer and Van Dam 1974; Conover 1987). The tricarboxylate carrier has been purified to homogeneity from liver mitochondria of yellow and silver eels and functionally reconstituted into liposomes (Zara et al. 1996; Zara et al. 2000). The kinetic characterization of eel liver tricarboxylate carrier, accomplished in proteoliposomes, revealed interesting differences with respect to the functional properties exhibited by the homologous protein from rat liver mitochondria (Zara et al. 1998). The transmembrane organization and oligomeric state of this transport protein have been investigated in eel liver mitochondria (Capobianco et al. 2002). Moreover, the role of cysteine residues has been studied using differently permeable sulphydryl reagents (Capobianco et al. 2004). In addition, the full-length cDNA and amino acid sequences of the eel citrate transporter were determined (Zara et al. 2007). In recent years, mitochondrial carriers have been mainly expressed in *Escherichia coli* where they accumulate as inclusion bodies (Palmieri 2004; Palmieri et al. 2006a). After disaggregation of the inclusion bodies the amount of renatured protein is sufficient for biochemical characterization (Palmieri 2004; Palmieri et al. 2006a). However, the expression and (re)folding conditions required for this process must often be tailored to the particular protein of interest. Also, these methods do not accurately represent natural folding pathways, leading to question whether these techniques will yield correct structures for the majority of membrane proteins (Wagner et al. 2006). Furthermore, expression in *E. coli* has sometimes resulted null or very low because of protein toxicity (Miroux and Walker 1996) or unfavorable codon usage (Sharp et al. 1988). The baculovirus expression system, developed by Smith (Smith et al. 1983, Smith et al. 1992), is an efficient way for overcoming the drawbacks described above and for producing high levels of recombinant membrane proteins (Eifler et al. 2007). In most cases, the recombinant membrane proteins in insect cells exhibited identical transport properties as in native tissues (Knecht et al. 1997).

In this study we report for the first time the overexpression of a mitochondrial carrier in a baculovirus system, i.e. overexpression of the mature eel liver CIC. Expression was optimised in terms of harvest time and multiplicity of infection (MOI). The cellular localization of the tagged protein was investigated using confocal laser microscopy. The pure recombinant protein was obtained employing a short purification procedure and yielded an active carrier with high specific activity. We show that the functional properties of the recombinant CIC are similar to those determined for the native carrier following isolation from eel liver mitochondria.

Materials and methods

Construction of the transfer plasmid

Total silver eel RNA from liver was extracted with a triazol kit (Invitrogen), following the supplier's protocol. 2.5 μg of total RNA were reverse-transcribed with the Gene Amp RNA PCR Core kit (PerkinElmer Life Sciences) using random hexamers as primers (final volume, 40 μl). The coding sequence of silver eel CIC (Zara et al. 2007) was amplified by PCR using primers 5'-TAGGGATCCGCCACCATGGCACCGGCAGGCA-3' (sense) and 5'-CGAGAATTCAGCTCAGTCTTCCA GACCTTGTTCAGAAC-3' (antisense). The product was cloned into the pBlueBac 4.5/V5 His baculo transfer vector and transformed into *E. coli* TG1 cells. The recombinant clones were selected on ampicillin (100 μg/ml), screened by colony PCR and restriction digestion of plasmids. The sequence of insert was verified. The encoded protein had additional C-terminal V5 epitope, followed by six histidines.

Insect cell transfection, recombinant virus isolation and protein expression

The insect ovarian cell, *Spodoptera frugiperda* (Sf9), the baculovirus transfer vector (pBlueBac 4.5/V5 His), baculovirus (Bac-N-Blue™ DNA) and Cellfectin® Reagent were purchased from Invitrogen. Sf9 cells were cultured at 27°C in *Trichoplusia ni* Medium-Formulation Hink (TMN-FH) basal medium (SIGMA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Recombinant baculovirus was generated by cotransfecting the insect cells (2×10^6 cells/ml) with 0.5 μg of the Bac-N-Blue™ DNA and 4 μg of recombinant transfer plasmid containing the coding sequence of silver eel CIC using the cationic lipid reagent Cellfectin. The culture media were collected 72 h after transfection, the virus in the supernatant was titrated using plaque assay tecnique (O'Reilly et al. 1992). The reconstitution of LacZ gene allowed for rapid detection of recombinant viral lysis blue plaques. Recombinant viruses were plaque-purified to eliminated wild-type virus as confirmed by PCR. High-titre virus stocks were prepared and stored at 4°C and used for infection.

For protein production Sf9 cells (3×10^6 cells/25 cm^2 flask) were infected with recombinant baculovirus at a multiplicity of infection (MOI) 10. After infection for 72 h, the cell pellets in lysis buffer [10% (w/v) SDS; 10 mM Tris-HCl pH 6.8] were incubated at 90°C and passed through an 18-gauge needle to shear the DNA. Recombinant proteins were analyzed by Western blotting using mouse anti-V5 monoclonal antibody.

Fixing and staining cells for immunofluorescence analysis

The procedure was performed as described earlier (Dolby et al. 2004). In brief, 1×10^6 Sf9 cells were grown on poly-L-lysine coverslips in 6-well plates and infected with the recombinant baculovirus at a MOI of 10. The infected cells were allowed to grow for 72 h. Then, the cells were incubated for 15 min at 27°C in the presence of 100 nM MitoTracker Red CMXRos (Molecular Probes, Leiden, The Netherlands), washed twice with PBS before fixing the cells in 1 ml of 3.7% (v/v) paraformaldehyde in PBS at room temperature for 15 min. The coverslips were washed twice with PBS. The cells were permeabilized and blocked with ice cold acetone for 10 min at room temperature. The cells were washed two times in PBS before adding a primary antibody against V5 (1 μg/ml in PBS) and incubated 60 min at room temperature. After washing three times 5 min with PBS, the secondary antibody anti-mouse FITC-conjugate was added at 1:500 dilution in PBS for 20 min at room temperature. In the final step, the coverslips were washed three times in PBS and the cells were visualised using by an inverted Zeiss Axiovert 200 motorized microscope (Zeiss, Jena, Germany) equipped with epifluorescence. Cells were imaged with a CoolSNAP HQ CCD camera (Roper Scientific, Trenton, NJ, USA) using the Metamorph software (Universal Imaging Corporation, Downingtown, PA, USA).

Preparation of mitochondria

All procedures were carried out at 4°C on ice. The insect cells were harvested by centrifugation at $1000 \times g$, washed in PBS and stored at −80°C. The frozen pellet deriving from 400 ml cell culture (1×10^6 cells/ml) was resuspended in 10 ml of lysis buffer [50 mM sodium phosphate buffer pH 7.8, 10% (w/v) glycerol, 10 mM β-mercaptoethanol, the following protease inhibitors (Roche) 1 mM PMSF, 2 mM Pepstatin A, 0.02 mM Leupeptin, 2 mM Benzamidine] and incubated on ice for 20 min. Cells were then lysed by 20 strokes in a Dounce homogenizer using a tight-fitting pestle (Loguercio Polosa et al. 2007). The omogenate was centrifuged at $1000 \times g$ for 10 min. The supernatant was centrifuged at $8000 \times g$ for 15 min. The pellet (mitochondrial fraction) was stored at −80°C until use.

Differential detergent solubilization of Sf9 mitochondria

Mitochondria recovered from 10 ml of culture (1.5 mg of protein) were resuspended in ice-cold solubilization buffer A (30 mM NaCl, 10 mM PIPES, pH 7.0 and protease inhibitors described above) supplemented with 2% TX-100 (w/v), or 2% DDM (w/v), or 2% OG (w/v) at a final concentration of 1.5 mg protein/ml. The suspension was mixed and incubated on ice at different times, (30 min, 2 h and overnight). The mitochondria pellet (insoluble materials) and the mitochondrial extract (soluble materials) were separated by centrifugation at $138000 \times g$ for 20 min at 4°C. Optimal solubilization conditions were determined for each detergent as described in results.

Purification of recombinant CIC/V5-His by Ni^{2+}- NTA agarose affinity chromatography

All purification steps were performed at 0–4°C on ice. 1.5 mg of mitochondria were solubilized with buffer A (100 mM NaCl and 10 mM Pipes pH 7.0) containing 2% TX-100 (w/v), at a final concentration of 1.5 mg protein/ml. After incubation for 30 min at 4°C with constant rotation, the insoluble materials were removed by centrifugation for 20 min at $138000 \times g$ for 20 min at 4°C. The supernatant was incubate batchwise for 20 min at 4°C with 0.4 ml Ni^{2+}-NTA agarose (Qiagen) previously equilibrated in buffer A. Subsequently, the resin was packed into a column (0.5 cm internal diameter) and unspecifically bound proteins were washed with the buffer A supplemented with 0.25 mg/ml of cardiolipin and increasing concentrations of imidazole (Palmieri et al. 2001). Pure CIC/V5-His was recovered with 1 ml of buffer B containing 100 mM NaCl, 100 mM imidazole, 0.4% TX-100 (w/v), 10 mM PIPES pH 7 and 4 mg/ml of cardiolipin. The purity of the protein sample was confirmed by SDS-PAGE and Western blot analysis.

Reconstitution into liposomes and transport assays

Purified recombinant protein was reconstituted into liposomes in the presence or absence of substrates (Palmieri et al. 1995). The reconstitution mixture contained solubilized proteins, 90 μl of 10% TX-114 (w/v), 90 μl of 10% phospholipids as sonicated liposomes (w/v), 20 mM citrate (except where indicated otherwise), 10 mM PIPES, pH 7, cardiolipin (0.5 mg, Sigma) and water to a final volume of 700 μl. These components were mixed thoroughly and the mixture was recycled 13 times through the same Amberlite column (Bio-Rad). 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 (Carrisi et al. 2008).

Other methods

Proteins were analysed by SDS-PAGE and stained with Coomassie Blue dye. The amount of pure CIC/V5-His 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 (Fiermonte et al. 1998). Western blot analysis was carried out as described previously using mouse anti-V5 monoclonal antibody (Capobianco et al. 1996).

Results

Expression of silver eel CIC/V5-His protein in insect cells

Pure recombinant virus was isolated by plaque assay and identified by PCR. Wild-type Bac-N-Blue virus showed a fragment of 839 bp corresponding to polyhedrin gene (Fig. 1A, lane 1), whereas the recombinant virus exhibited a band of about 1.4 kb (Fig. 1A, lane 2) corresponding to the transcript of mature silver eel CIC cloned into the pBlueBac4.5/V5-His vector. Cells infected with purified recombinant CIC/V5-His virus produced a new protein that was detected when cells were directly lysed in denaturing buffer and subjected to SDS-PAGE and Western blot. The mouse anti-V5 monoclonal antibody immunodecorated a single band with an apparent molecular mass of about 34 kDa only in eel CIC/V5-His-infected cells (Fig. 1B, lane 2). To optimize the yield of recombinant CIC/V5-His, a combination of two parameters was evaluated including MOI and post-infection time. Sf9 cells were infected at cells densities of 1×10^6/ml at MOI of 5, 7 or 10 and at post-infection time (24 h, 48 h, 72 h, 96 h and 120 h). Samples were analysed by Western blot (Fig. 2). The recombinant CIC/V5-His protein became immunodetectable within 48 h (Fig. 2, lanes 7–9) and protein expression was observed to increase up to 72 h after infection (Fig. 2, lanes 10–12); at 72 h post-infection, however, the amount of expressed protein began to decrease (Fig. 2, lanes 13–18). The recombinant CIC/V5-His protein reached a peak in 72 h post-infection and the optimal MOI was 10. We therefore used MOI 10 and 72 h post-infection cells in additional experiments. Cell lysates infected with wild-type uncut Bac-N-Blue virus as control did not express immunodetectable proteins (data not shown).

Subcellular localization of the CIC/V5-His protein

Intracellular accumulation of membrane proteins is a common observation in overexpression studies (Winkler et al. 2001; Goffart et al. 2007). To investigate the precise intracellular localization of the recombinant CIC/V5-His protein immunofluorescence studies were performed (Fig. 3). After 72 h of infection CIC/V5-His-expressing cells exhibited green fluorescence (Fig. 3, panel A). Upon staining with the mitochondrial-specific dye, MitoTracker Red, the same cells showed a pattern of fluorescence that coincides with the mitochondrial network (Fig. 3, panel B). From the overlapping images it is clear that recombinant CIC/V5-His protein was localized to mitochondria (Fig. 3, panel C). Structural integrity of the cells was documented by phase contrast microscopy (data not shown).

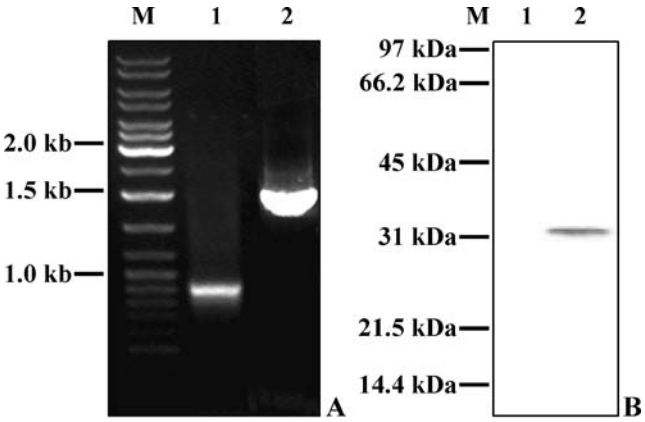

Fig. 1 Recombinant silver eel CIC/V5-His protein expression in the baculovirus system. **A** Isolation of pure CIC/V5-His recombinant virus. Lane M, 1 kb DNA ladder marker; lane 1, wild-type uncut Bac-N-Blue virus showing the 839 bp band of the polyhedrin gene; lane 2, pure recombinant CIC/V5-His virus showing the 1419 bp band corresponding to the silver eel CIC cDNA. The analysis was done by PCR of DNA isolated using the plaque assay. **B** Identification of recombinant silver eel CIC/V5-His protein. Sf9 cells infected with recombinant CIC/V5-His virus were analyzed by SDS-PAGE and Western blot using mouse anti-V5 monoclonal antibody. Lane M, marker (Phosphorylase b, Serum albumin, Ovalbumin, Carbonic anhydrase, Trypsin inhibitor and Lysozyme); lane 1, cell extract from Sf9 cells infected with wild-type Bac-N-Blue virus; lane 2, cell extract from Sf9 cells infected with pure recombinant CIC/V5-His virus. The relative position of the molecular mass markers is indicated on the left

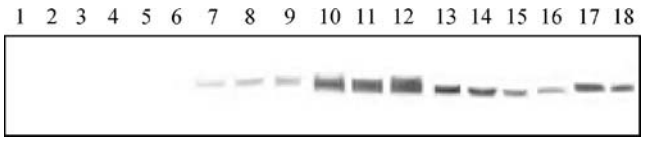

Fig. 2 Optimization of the recombinant protein expression. Time course of expression kinetics for recombinant CIC/V5-His protein in Sf9 cells. The cells were infected with a recombinant baculovirus at MOI of 5, 7 and 10 respectively. At different times post-infection, cells were harvested and subjected to immunoblotting. Lysates of Sf9 infected cells (50μg/lane) were separarted by SDS-PAGE and transferred to nitrocellulose membranes. Immunoblot analyses of the transferred protein were performed using mouse anti-V5 monoclonal antibody. Lanes 1–3: at 0 h with MOI 5, 7 and 10 respectively; lanes 4–6 at 24 h with MOI 5, 7 and 10 respectively; lanes 7–9 at 48 h with MOI 5, 7 and 10 respectively; lanes 10–12 at 72 h with MOI 5, 7 and 10 respectively; lanes 13–15 at 96 h with MOI 5, 7 and 10 respectively; lanes 16–18 at 120 h with MOI 5, 7 and 10 respectively

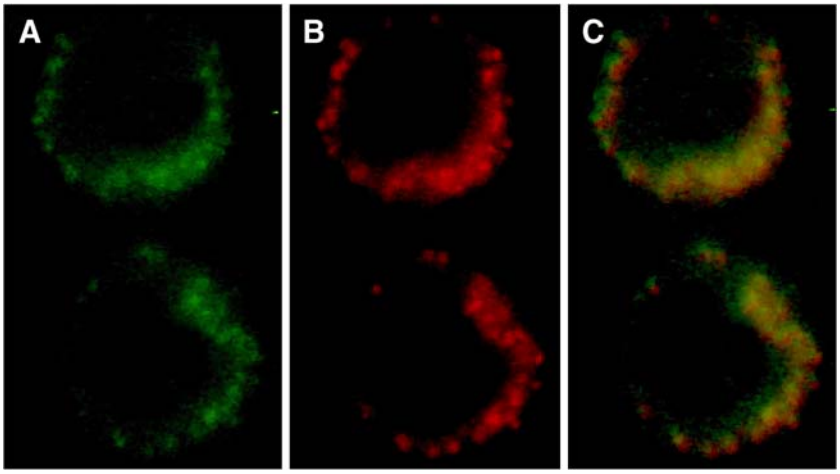

Fig. 3 Subcellular localization of the recombinant CIC/V5-His protein after expression in Sf9 insect cells. Sf9 insect cells over-expressing CIC/V5-His protein were grown as described in Experimental section. Staining of CIC/V5-His protein was performed with mouse anti-V5 monoclonal antibody and anti-mouse antibody with FITC-conjugated (Panel A). MitoTracker Red was used to locate mitochondria in the cells (Panel B). Colocalization of the CIC/V5-His protein and mitochondria is seen as yellow fluorescence in the red and green merged image (Panel C). The same cells were photographed first with a FITC-green filter set and then with the MitoTracker red-filter set. Identical fields are presented

Differential detergent solubilization of the CIC/V5-His protein

Detergents are critical to the isolation and solubilization of membrane proteins, and the selection of suitable detergents is an important aspect of successful functional membrane protein purification.

The non-ionic detergents TX-100, DDM and OG were tested for solubilization of the CIC/V5-His protein. TX-100 was found to be effective in the purification of the mitochondrial CIC protein (Bisaccia et al. 1989), whereas DDM and OG were found to be effective for solubilization of the recombinant protein from Sf9 membranes (Mitic et al. 2003; Hung et al. 2007). Identical amounts of mitochondria were treated for 30 min at 4°C with 2% TX-100, 2% DDM and 2% OG and cleared by ultracentrifugation of the solubilized samples. Western blot analysis performed on the mitochondrial extract (Fig. 4A, lanes 1, 3 and 5) and pellet (Fig. 4A, lanes 2, 4 and 6) utilizing mouse anti-V5 monoclonal antibody showed that TX-100 and DDM were the most effective reagents in solubilizing the recombinant CIC, whereas OG was ineffective and no protein recovery was observed. Moreover, no increase of extracted protein content was observed when mitochondria were solubilized for 2 h or overnight (data not shown). Furthermore, for each mitochondrial extract, transport activity of the CIC/V5-His protein was evaluated (Fig. 4B). The highest transport activity was found using 2% TX-100 while DDM led to a transport activity of about 50% compared to TX-100; virtually no citrate exchange activity was measured in the extracts obtained by using OG. In additional experiments, transport activity was reduced by

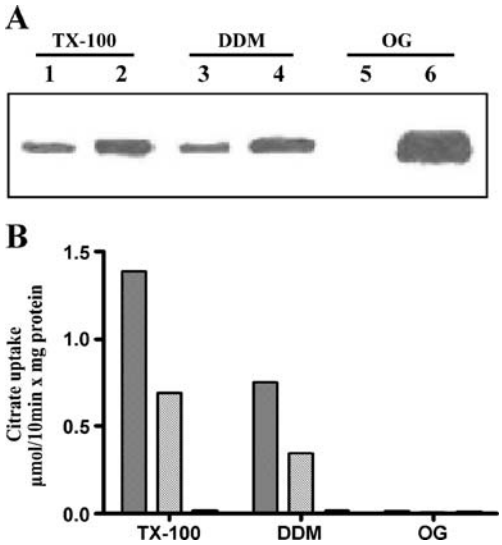

Fig. 4 Differential non-ionic detergent solubilization of recombinant CIC/V5-His protein from Sf9 mitochondrial membranes. **A** Western blot analysis of mitochondrial membrane proteins (40 µg) solubilized for 30 min in presence of 2% TX-100, DDM and OG as described in Materials and methods. The mitochondrial extracts and pellets were separated by SDS-PAGE, transferred to nitrocellulose and immunodecorated with mouse anti-V5 monoclonal antibody. Lanes 1, 3 and 5: mitochondrial extracts; lanes 2, 4 and 6 mitochondrial pellets. **B** Transport activity of the recombinant CIC/V5-His protein extracted at 30 min (hatched bars), 2 h (dotted bars) and overnight (black bars) in the presence of non-ionic detergents. Transport was started by adding 0.5 mM [^{14}C]citrate to proteoliposomes reconstituted with recombinant CIC/V5-His protein and stopped after 10 min. Similar results were obtained in at least three independent experiments

approximately 50% when mitochondria were solubilized for 2 h with TX-100 or DDM, and a total loss of transport activity was observed when mitochondria were solubilized overnight (Fig. 4B). The mitochondria of non-infected Sf9 cells, solubilized with the same detergents and times, exhibited very low citrate transport activity (data not shown). The results obtained led us to carry out mitochondrial protein extraction using TX-100 at 2% for 30 min.

Purification of the recombinant CIC/V5-His protein and comparison with eel liver mitochondria citrate carrier

Cells grown and infected in small spinner flasks were used to isolate the recombinant CIC. The results are documented in Fig. 5 and in Table 1. Mitochondria (1.5 mg) were recovered from 10×10^6 cells; the recombinant protein was extracted from mitochondrial membranes using 2% TX-100 as described in Material and Methods. The recombinant CIC/V5-His protein was purified from mitochondrial extract by affinity chromatography on a Ni^{2+}-NTA chelating column. The protein was recovered with 1 ml of 100 mM imidazole; the eluate obtained contained a homogeneous protein with an apparent molecular mass of 34 kDa (Fig. 5A, lane 5) corresponding to that of the CIC/V5-His protein. Protein identity was confirmed by Western blot analysis (Fig. 5B, lane 5). About 0.4–0.5 mg of purified protein was obtained per liter of culture. The specific citrate transport activity of our purified preparation was enhanced 55-fold compared to that of the mitochondrial extract of infected cells expressing the recombinant protein (Table 1). Approximately 22% of the total citrate transport activity applied to the column was recovered with a yield of 0.39%.

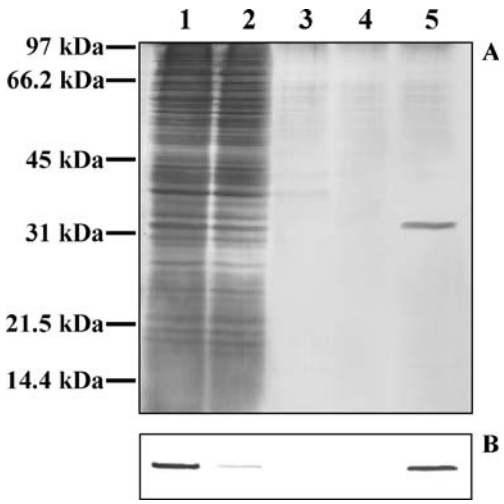

Fig. 5 Purification of the recombinant CIC/V5-His protein by Ni^{2+}-NTA agarose affinity chromatography. Proteins were separated by SDS-PAGE and stained with silver nitrate (A) or transferred to nitrocellulose and immunodecorated with mouse anti-V5 monoclonal antibody (B). Lanes M, markers (Phosphorylase b, Serum albumin, Ovalbumin, Carbonic anhydrase, Trypsin inhibitor and Lysozyme); Mitochondrial extract (Panels A and B, lane 1); pass through (Panels A and B, lane 2); column washes (Panels A and B, lanes 3–4); purified recombinant CIC/V5-His protein (Panels A and B, lane 5)

Functional characterization of the recombinant citrate carrier

In the experiments described in this section, the recombinant purified CIC/V5-His protein was reconstituted into liposomes and its transport properties were studied by measuring the uptake of external [^{14}C]citrate into proteoliposomes preloaded with unlabeled citrate. The purified CIC/V5-His recombinant protein reconstituted into liposomes catalyzed a counter-exchange of 0.5 mM external [^{14}C]citrate for 20 mM internal citrate with first order kinetics (rate constant, 0.097 min^{-1}), isotopic equilibrium being approached exponentially (data not shown). The exchange reaction was completely inhibited by addition of 20 mM 1,2,3-BTA. Maximum uptake of [^{14}C]citrate was approached after 20 min. The corresponding value at infinite time was 129.5 µmol/mg protein. The initial rate of citrate uptake (the product of *k* and intraliposomal quantity of citrate taken up at equilibrium) was 12.5 µmol/min×mg protein. In proteoliposomes lacking the substrate, or if the solubilized protein was boiled prior to incorporation into liposomes, no uptake of labeled external substrate into proteoliposomes was observed. To further assess the identity of the recombinant CIC/V5-His protein, its activity has been investigated in proteoliposomes preloaded with various substrates. As shown in Table 2, [^{14}C]citrate was efficiently taken up by proteoliposomes containing threo-isocitrate, phosph*enol*pyruvate and L-malate. Virtually no exchange was observed with internal substrates of other mitochondrial carriers such as phosphate, 2-oxoglutarate, glutamate and ATP. The [^{14}C]citrate/citrate exchange reaction catalyzed by the reconstituted CIC/V5-His protein was strongly inhibited by the impermeable-specific inhibitor 1,2,3-BTA (but only slightly by its structural isomers 1,2,4-BTA and 1,3,5-BTA) and by BAT, PLP and thiol reagents (*p*-hydroxymercuribenzoate, mersalyl and mercuric chloride). Carboxyatractyloside, a specific and powerful inhibitor of the mitochondrial ADP/ATP carrier (Kramer and Klingenberg 1979), had no effect on the activity of the recombinant CIC/V5-His protein. The kinetic constants of the recombinant purified CIC/V5-His protein 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 68±0.1 µM and 14.2±1.7 µmol/min x mg protein, respectively (5 experiments). The activity was calculated by taking into account the amount of CIC/V5-

Table 1 Purification of citrate carrier from mitochondria of Sf9 infected cells

Fraction	Protein (mg/ml)	Specific activity	Total activity	Purification (fold)
Sf9 non-infected extract	1.57	0.03	62.3	–
Sf9 infected extract	1.32	1.4	1895.5	–
Ni^{2+}-NTA eluate	0.005	78.9	410.6	55

Extract refers to mitochondria isolated from Sf9 non-infected and infected cells with recombinant *Autographa calfornica* nuclear polyhedrosis virus containing the CIC cDNA and extracted with 2% Triton X-100. Ni^{2+}-NTA eluate refers to the active CIC/V5-His protein purified by affinity chromatography. The proteoliposomes were preloaded internally with 20 mM citrate and the exchange was started by adding 0.5 mM [^{14}C]citrate. The activity of the reconstituted citrate exchange is expressed as μmol/10 min×mg protein (specific activity) and nmol/10 min × ml (total activity). The values are means of four experiments.

His protein recovered in the proteoliposomes after reconstitution, as described in Materials and Methods. Taken together, the transport properties, substrate specificity, inhibitor sensitivity and kinetic characteristics of the recombinant CIC/V5-His protein reconstituted in phospholipid vesicles clearly demonstrate that it corresponds to the mitochondrial tricarboxylate carrier previously characterized from silver eel liver mitochondria (Zara et al. 1996, 1998, 2000).

Discussion

The majority of membrane transport proteins, as in the case of the inner mitochondrial CIC, are present in native tissues at very low concentrations providing insufficient material for biochemical and biophysical investigations. One approach to overcoming this problem is the overexpression of the recombinant protein in a functional state (Grisshammer and Tate 1995).

In the past, many mitochondrial carrier proteins from man, *Saccharomyces cerevisiae* and *Arabidopsis thaliana* have been expressed in large amounts in *E. coli* (Palmieri 2004; Picault et al. 2004; Palmieri et al. 2006a, b; Palmieri et al. 2008). This latter organism is an attractive expression system because it is easy to scale up and inexpensive to grow; however, there are some drawbacks associated with its prokaryotic nature. The low percentage of GC nucleotides in the genome and/or the existence of rare codons often result in low or no detectable protein expression (Miroux and Walker 1996; Fiermonte et al. 1993; Heimpel et al. 2001). Heterologous expression in bacteria also suffers from the inability of prokaryotes to perform post-translational modifications (e.g., glycosylation, fatty acid

Table 2 Substrate specificity and inhibitor sensitivity of the recombinant CIC/V5-His protein in reconstituted liposomes

Internal substrate (20mM)	Inhibitor	Exchange activity (%)
Citrate		100
threo-Isocitrate		103
Phospho*enol*pyruvate		115
L-Malate		97
Phosphate		1
2-Oxoglutarate		2
Glutamate		1
ATP		1
Citrate	Mersalyl (0.1 mM)	1
Citrate	*p*-hydroxymercuribenzoate (0.1 mM)	1
Citrate	$HgCl_2$ (0.1 mM)	1
Citrate	BAT (10 mM)	1
Citrate	PLP (10 mM)	15
Citrate	1,2,3-BTA (2 mM)	4
Citrate	1,2,4-BTA (2 mM)	74
Citrate	1,3,5-BTA (2 mM)	69
Citrate	Carboxyatractyloside (5 mM)	93

Proteoliposomes were preloaded internally with the indicated substrates and the exchange was started by adding 0.07 mM [^{14}C]citrate. BAT, PLP, 1,2,3-BTA, 1,2,4-BTA, 1,3,5-BTA and carboxyatractyloside were added simultaneously with [^{14}C]citrate, whereas the SH reagents were added 2 min before the labelled substrate. The activity of the citrate/citrate exchange was 7.24 μmol/min × mg protein. The values are means of four experiments.

acylation, phosphorylation), some of which are known to be critical for several membrane proteins (Shi and Jarvis 2007). In addition, overexpression of mitochondrial membrane proteins results in sequestration of the protein in insoluble aggregates, known as inclusion bodies (Fiermonte et al. 1993), that need to be disaggregated and renatured. Furthermore, membrane proteins have proven to be particularly resistant to in vitro (re)folding strategies, resulting in an inactive protein not useful for biochemical characterization. On the contrary, the baculovirus system is an excellent method for producing sufficient quantities of eukaryotic refolded recombinant proteins; in addition, it can affect post-translation modification and translocation of the membrane proteins in the appropriate subcellular compartment (Kost et al. 2005).

This is the first investigation in which the Sf9-baculovirus system has been used to express a mitochondrial carrier protein with the aim of obtaining an active protein. The system has been optimized to obtain baculoviruses that infect host cells with great efficiency, producing a large population of cells that express the protein of interest (Fig. 2). Furthermore, since overexpression of a membrane protein often leads to accumulation of recombinant non-native protein in extramembranous particles (Winkler et al. 2001; Goffart et al. 2007), the subcellular localization has been investigated. Our results, obtained with the recombinant CIC/V5-His protein, provide evidence that even at high expression levels our protein is transferred exclusively to the mitochondria of insect cells, demonstrating that this system is able to target the recombinant CIC/V5-His protein to the appropriate cellular compartment (Fig. 3).

Solubilization is a critical and essential step in membrane protein purification, which may destabilize the carrier and result in time-dependent loss of functional properties (Rosenbusch 2001; Ratnala et al. 2004). Optimum conditions to extract the recombinant protein have been achieved by evaluating the solubilization time and transport activity utilizing TX-100, DDM and OG, three different detergents found to be effective for the solubilization of membrane proteins (Bisaccia et al. 1989; Mitic et al. 2003; Hung et al. 2007). In agreement with the results obtained for other mitochondrial carriers (Palmieri et al. 1993), ours demonstrate that TX-100 is the most effective detergent for solubilizing recombinant CIC/V5-His protein from insect mitochondria (Fig. 4).

To evaluate the extent of CIC/V5-His protein expression in insect cells, its activity was compared in mitochondrial extracts from various origins reconstituted in proteoliposomes. The specific activity was 0.04 µmol/10 min × mg protein in eel liver mitochondria (Zara et al. 1996), 1.4 µmol/10 min × mg protein in mitochondria of infected cells expressing the recombinant carrier, and 0.03 µmol/10 min x mg protein in mitochondria of non-infected insect cells (Table 1). The ratio between the citrate transport activities of mitochondria from infected insect cells and the eel liver mitochondria was 34:1 and the ratio between the activities of mitochondria from infected insect cells and the mitochondria from non-infected insect cells was about 36:1. These results are in agreement with the high expression levels of CIC/V5-His protein in insect cells.

Rapid, one-step purification of the CIC/V5-His protein was achieved; the purity and integrity of the purified protein were monitored by SDS-PAGE, Western blot analysis and transport activity. Protein-stained gel reveals a major band at 34 kDa that is detected with mouse anti-V5 monoclonal antibody and therefore represents the intact protein (Fig. 5). The specific citrate transport activity of our purified preparation was enhanced about 55-fold compared to that of the mitochondrial extract of infected cells (Table 1). It is worth noting that the transport activity of our purified protein is higher than that previously reported for the purified eel liver CIC (Zara et al. 1996), most likely because the multistep protocol, used to purify the protein from tissue, could have caused partial loss of protein activity. Moreover, our data show that the purified recombinant CIC/V5-His protein has retained the substrate specificity, inhibitor sensitivity (Table 2) and kinetic characteristics that have been previously demonstrated for the native eel liver CIC (Zara et al. 1996).

To our knowledge, this is the first report on baculovirus-mediated production of a mitochondrial carrier protein in insect cells. This strategy, which combines expression in a heterologous system with protein purification by affinity chromatography, provides a source of highly pure protein in amounts sufficient for functional characterization and structural/biophysical studies. Due to the structural similarities between the CIC and other members of the mitochondrial carrier family (Palmieri 2004; Palmieri 2008), it is likely that the same protocol successfully applied to the CIC can be employed for the expression and purification of other mitochondrial transporters that are currently expressed in *E. coli* with low or null amounts of renaturated protein. Furthermore, the baculovirus system can be a good alternative to express those membrane proteins that are toxic or have an unfavorable bias for expression in *E. coli*.

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