Bacterial over-expression of functionally active human CT2 (SLC22A16) carnitine transporter

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Short Report

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Abstract

I. Background: *E. coli* is a widely used tool for the over-expression of human proteins for studying structure and function. The toxicity of human proteins for *E. coli* often hampers the expression. This study aims to find conditions for the expression of a membrane transporter known as the carnitine transporter CT2. The knowledge on this transporter is scarce, thus obtaining the recombinant protein is very important for further studies.

II. Methods and Results: The cDNAs coding for human CT2 (hCT2) was cloned in the pH6EX3 vector and different transformed *E. coli* strains were cultured in absence or in presence of glucose. hCT2 expression was obtained. The protein was purified and reconstituted into proteoliposomes in a functionally active state.

III. Conclusions: using the appropriate IPTG concentration, together with the addition of glucose, hCT2 has been expressed in *E. coli*. The protein is active and shows capacity to transport carnitine in proteoliposomes. The results have a great interest in basic biochemistry of membrane transporters and applications to human health since hCT2 is involved in human pathology.

Introduction

Carnitine is a vitamin-like molecule involved in the transport of long-chain fatty acids from the cytosol to the mitochondria, where they are metabolized through β -oxidation [1]. In humans, the mitochondrial transport of carnitine derivatives is mediated by the carnitine/acylcarnitine translocase (SLC25A20) [2], whereas the absorption, re-adsorption and tissue distribution occur via some members of the SLC22 family [3, 4], among which, the members number 5 (SLC22A5) and 16 (SLC22A16) are those mainly responsible for carnitine traffic in human body. In particular, SLC22A5, known as OCTN2, is an high affinity transporter for carnitine, thus playing a pivotal role in the carnitine homeostasis maintenance, being mainly localized at the intestinal and renal epithelia [5]. The SLC22A16, known as CT2, FLIPT2, or OCT6, is described as a carnitine transporter with a peculiar tissue distribution; indeed, the protein is particularly expressed in the bone marrow, in testis, where it is involved in the maturation of human spermatozoa [6], in hematopoietic tissues, including CD34+ and leukemia cells [7], and in endometrium where its expression is regulated by progesterone [8]. The studies on SLC22A5 are at a more advanced stage of investigation, whereas, the knowledge on SLC22A16 is still at infancy. As a carnitine transporter, SLC22A16 is involved in the regulation of fatty acid oxidation (FAO), which plays an important role also in the metabolic transformation of cancer cells [9]. In particular, AML (Acute Myeloid Leukemia) cells strongly depends on mitochondrial metabolism and FAO and present a specific up-regulation of the SLC22A16 carnitine transporter which can be exploited as a pharmacological target [10]. Indeed, SLC22A16 inhibition reduced the growth/viability and cell cycle progression of AML cells and enhanced the cytotoxic effect of chemotherapeutic agents, including daunorubicin, cytarabine, tigecycline or etomoxir [10]. Due to its involvement not only in carnitine traffic, but also in drugs absorption in healthy and cancer cells, SLC22A16 can be considered as a multirole transporter [11], in line with the main

features of the SLC22 family members. As an example, the SLC22A16 over-expression in different cancer cell lines [12, 13], drove the characterization of SLC22A16 as a doxorubicin importer. Therefore, several studies aimed to investigate the effect of SLC22A16 genotype on doxorubicin pharmacology. In particular, the SLC22A16 T1226C was associated with a greater incidence of “doxorubicin dose delay” and leucopenia but had no impact on survival on 230 patients recruited at Newcastle Tyne Hospitals NHS Foundation Trust [14], whereas the c.146A>G variant may influence the pharmacokinetics of doxorubicin and doxorubicinol in asian breast cancer patients [15], and rs12210538 A/G may increase the probability of 3/4 febrile neutropenia in iranian breast cancer patients [16]. Moreover, SLC22A16 mediates cisplatin uptake in lung cancer cells [17], and high-affinity uptake of the anticancer polyamine analog bleomycin-A5 in colorectal cancer cells [18]. In this scenario, the availability of the recombinant human SLC22A16 transporter may have a great relevance for performing structure/function relationships studies as well as applications to human health, allowing directly testing interaction of candidate drugs with the transporter. We report the successful over-expression of the recombinant human SLC22A16 using a strategy based on appropriate modulation of inducer concentration, growth temperature, and glucose supply to bacterial cells. Interestingly, the over-expressed protein was obtained in a functionally active form, that opens important perspectives for addressing the above-described purposes.

Materials And Methods

Chemicals used for experiments, protease inhibitor cocktail (P8849), Nickel Affinity Gel (HIS-Select® - P6611) and the Monoclonal Anti-polyHistidine-Peroxidase antibody (A7058) were from Sigma–Aldrich; pCOLD I plasmid from Takara; restriction endonucleases and specific reagents for cloning from Thermo scientific; *E. coli* RosettaGami2 strain from Novagen; BL21 codon plus RIL and Lemo21 from Agilent technologies; codon optimized hCT2 sequence was from Genscript. L-[³H]-Carnitine from ARC (America Radiolabeled Chemicals), C₁₂E₈ from TCI Europe, Amberlite XAD-4, egg yolk phospholipids, Sephadex G-75, L-carnitine from Sigma-Aldrich.

Cloning of hCT2

The cDNA encoding for human CT2 transporter (SLC22A16) (UniProtKB: Q86VW1; GenPept accession no. NP_149116.2), was codon optimized according to the *E. coli* codon usage by Genscript and cloned between *Bam* HI and *Sa*/I restriction sites. The 1734 bp cDNA corresponding to the hCT2 encoding sequence was then sub-cloned, by double digestion, into the same restriction sites of the pH6EX3 or pCOLD I expression vector. The resulting recombinant plasmids, defined as pH6EX3-hCT2 and pCOLD I-hCT2, encode a 6His-tagged fusion protein corresponding to the hCT2 carrying the extra N-terminal MSPHHHHHHHLVPRGS or MNHKVHHHHHHIEGRHMELGTLGGS sequence, respectively.

Expression of hCT2 transporter

To produce the 6His-hCT2 recombinant protein, *E. coli* BL21 codon plus cells, were transformed with the pH6EX3-hCT2 or pCOLD I-hCT2 construct. Selection of transformed colonies was performed on LB-agar

plates added with 100 µg/mL ampicillin and 34 µg/mL chloramphenicol. Different media (LB or TY 2X at pH 7.0) prepared in absence or in presence of different glucose concentrations were assayed. According to the number of conditions to be tested, colonies were inoculated in a different volume of a specific medium and cultured overnight at 37 °C under rotary shaking (160 rpm). The day after, the culture was diluted 1:20 in fresh medium added with the specific antibiotics and specifically treated, according to the used construct. In the case of pCOLDI-hCT2 construct, when the optical density measured at 600 nm wavelength was 0.8-1, the cultures were shocked on ice for 10 minutes (under manual agitation every 1 minute), then incubated for 40 minutes at 15 °C under rotary shaking (160 rpm). Different IPTG concentrations (from 0.05 to 0.4 mM) were tested to induce protein expression with the exception of one aliquot, grown in absence of inducer, (negative control). The cultures were continued for up to 24 hours at 15 °C at 160 rpm. Every two hours, aliquots were collected and centrifuged at 3,000 x g, and at 4 °C for 10 minutes; the pellets were stored at -20 °C. A bacterial pellet aliquot, after thawing, was dissolved in a resuspension buffer (20 mM Hepes/Tris, 300 mM NaCl pH 7.5) added with protease inhibitor cocktail according to manufacturer instructions. The bacterial suspensions were sonified in an ice bath for 10 minutes (pulse of 1 second on, and 1 second off) at 40 Watt, using a Vibracell VCX-130 sonifier. The insoluble cell fractions were analyzed by SDS-PAGE and western blotting. In the case of pH6EX3-hCT2 construct, when the optical density measured at 600 nm wavelength was 0.8-1, the growth temperature was lowered to 28 °C and different IPTG concentrations (from 0.05 to 0.4 mM) were tested to induce protein expression with the exception of one aliquot, grown in absence of inducer, (negative control). The induced cultures and the control were kept at 28 °C under rotary shaking for up to 8 hours. Every two hours, aliquots were collected and treated as described above.

Purification of the hCT2 protein

In order to purify 6His- hCT2 protein, the pellet of the bacterial suspension containing the pH6EX3-hCT2 construct was washed with 0.1 M Tris/HCl pH 8.0, and centrifuged at 12,000 x g for 10 minutes. The new pellet was solubilized adding 400 µl of 8 M urea, 100 µl of 100 mM DTE, 54 µl of 10 % sarkosyl, and 546 µl of a renaturing buffer (A) containing: 0.1 % sarkosyl, 200 mM NaCl, 10% glycerol, and 20 mM Tris/HCl at pH 8.0. After centrifugation (12,000 x g, 10 minutes, 4 °C), the supernatant was added to a His select Ni²⁺ affinity gel column (0.5 x 7.5 cm; 3 mL= 1 Column Volume) equilibrated with 10 column volumes of a renaturing buffer A, and the mixed over night at 4°C in a fixed angle rotator for tubes. The day after, the protein resin mix (about 15 mL) was transferred in a column and packed by gravity. 10 mL washing buffer (0.3 % C₁₂E₈, 200 mM NaCl, 10% glycerol, 5 mM DTE, 20 mM Tris/HCl at pH 8), 25 mL washing buffer added with 15 mM imidazole, 7 mL of washing buffer added with 500 mM imidazole were used for eluting proteins in 42 fractions (1 mL). Purified hCT2 protein was mainly present in the fractions 39-40. The fractions were pulled together and passed through a PD-10 column for removing imidazole using a buffer composed by 0.3 % C₁₂E₈, 200 mM NaCl, 10% glycerol, 5 mM DTE, 20 mM Tris/HCl at pH 8. The protein was then used for reconstitution in proteoliposomes as described in the following paragraph.

Reconstitution of the hCT2 into liposomes

The purified hCT2 was reconstituted by removing the detergent from mixed micelles containing detergent, protein and phospholipids by incubation with 0.5 g Amberlite XAD-4 resin under rotatory stirring (1200 rpm) at 23°C for 60 min, using a procedure previously described [19]. The composition of the initial mixture used for reconstitution was: 350 µL of the purified protein (9 µg protein in desalting buffer), 70 µL of 10% C₁₂E₈, 100 µL of 10% egg yolk phospholipids in the form of sonicated liposomes prepared as previously described [20] and 20 mM Tris/HCl pH 7.5 in a final volume of 700 µL.

Transport measurements

To remove the external compounds, 600 µL of proteoliposomes were passed through a Sephadex G-75 column (0.7 cm diameter×15 cm height) pre-equilibrated with 10 mM Tris/HCl pH 7.5. From these columns, 550 µL of proteoliposomes were collected and divided into aliquots (samples) of 100 µL. Transport was started by adding 50 µM of [³H]-carnitine to the proteoliposome samples, as indicated in the figure legends. In control samples, transport in empty liposomes, i.e., without incorporated protein, is measured. The assay temperature was 25°C. Transport was stopped by passing 100 µL of each sample through a Sephadex G-75 column (0.6 cm diameter×8 cm height) in order to separate the external from the internal radioactivity. Samples were eluted with 1 mL 50 mM NaCl and collected in 3 mL of scintillation mixture, vortexed and counted. The experimental values were corrected by subtracting the respective control to obtain specific radioactivity expressed as nmol min⁻¹ mg protein⁻¹. Kinetic analysis was performed at 15 min, i.e., in the linear range of time courses. Experimental data were plotted using the Grafit (version 5.0.13) software using first order rate, Lineweaver–Burk equations. Results were expressed as means ± SD.

Data analysis

Results are expressed as means ± SD. GraFit 5.0.13 software was used to calculate kinetic parameters. Comparisons between two groups were performed with the two tailed Student's unpaired t test. For multiple comparisons, one-way ANOVA followed by Tukey's post hoc test was employed.

Protein quantification

Protein concentration was measured by the method of Lowry, modified for the presence of detergents [21]. Proteins were separated by SDS–PAGE on 12% polyacrylamide gels performed according to Laemmli [22], using the Hoefer SE260 mini-vertical unit and stained by Coomassie-brilliant blue. Quantitative evaluation of Coomassie-stained protein bands was carried out using the Chemidoc imaging system equipped with Quantity One software (Bio-Rad).

Western blot assay

hCT2 was immuno-detected using the Monoclonal Anti-polyHistidine-Peroxidase antibody 1:10,000.

Results And Discussion

Over-Expression of hCT2

In order to obtain expression of CT2, the hCT2-pH6EX3 or hCT2-pCOLD I construct was firstly used for transforming *E. coli* BL21 codon plus strain adopted to overcome codon bias. A single colony was inoculated from each different plate and cultured overnight in an appropriate volume of LB broth. After 12-16 hours, a 1: 10 dilution in fresh LB was performed and the growth was monitored. During the exponential phase of growth ($OD \sim 0.8$), the cultures containing the different constructs were differently treated as described in materials and methods section. Then, the IPTG concentration was modulated in a range of 0.05 to 0.4 mM to find the best compromise between optimal rate of protein synthesis and lowest toxic effects on bacteria. The production of the protein as function of time was then monitored in the case of transformation with hCT2-pH6EX3. Following IPTG addition, an OD reduction was observed in any condition with the maximum effect at the highest IPTG concentration tested (Fig. 1), highlighting a putative toxic effect of the protein on cell growth, as previously observed for other proteins [23]. Whereas, in the case of pCOLD I, the cells were harvested at a single time since growth was performed at low temperature, as suggested for this specific plasmid, leading to a very slow rate of growth in all conditions. The production of the protein, verified by western blotting analysis (Fig. 2a), was strongly influenced by the plasmid type indicating the pH6EX3 as the preferred plasmid (Fig. 2a lanes 1-6) with respect to pCOLD I (Fig. 2a, lanes 7-9). For the hCT2-pH6EX3 construct, the protein synthesis increased up to 8 hours (Fig. 2a, lanes 4-6) and was inversely proportional to the inducer concentration. This phenomenon, previously described in the case of other transporters [23], is in agreement with the toxicity of the protein of interest towards bacterial cells. Thus, the lower IPTG concentration resulting in a slower rate of protein production has the effects of decreasing the toxic effect. Surprisingly, the use of codon optimized construct did not improve the production of the protein of interest in these cells (not shown). Probably, the human tRNAs present in BL21 codon plus *E. coli* strain was enough to overcome the codon bias. Moreover, we also tested the possible effect of codon optimized construct transforming another *E. coli* strain, i.e., Lemo21 which does not carry human tRNAs, without any significant improvement (Fig. 2b). We could not well explain this phenomenon that might also be related with the relatively low rate of synthesis of the protein of interest. To further decrease the toxic effects due to a possible leakage expression of the protein of interest, the effect of glucose in the growth medium was tested. BL21 codon plus *E. coli* strain was transformed with hCT2-pH6EX3 construct and cultured as described above in absence or in presence of two different glucose concentrations (0.5% or 1%). The production of hCT2 increased with the increase of glucose concentration, being about four times higher in the presence of 1% glucose (Fig. 2c lane 5) than in the absence of glucose (Fig. 2c lane 1). Also in these conditions, the amount of over-expressed protein was inversely proportional to the inducer concentration.

Purification of over-expressed hCT2

Starting from the insoluble fraction of BL21 cell lysate obtained in optimal conditions, i.e., 8h of induction with 0.05 mM IPTG in presence of 1% glucose at 28°C, the purification of hCT2 protein was attempted under denaturing conditions followed by an on-column refolding procedure, as previously described for other SLC transporters [23, 24] with substantial modifications. For refolding, the strong detergent sarkosyl

was substituted by octaethylene glycol monododecyl ether (C₁₂E₈). The binding of hCT2 to the resin was effective, as low reactivity against anti-His antibody was observed in the corresponding pass-through fraction (Fig. 3 lane 8). After washing with 10 mM imidazole (Fig. 3, lane 4), hCT2 was eluted by 500 mM imidazole (Fig. 3, lane 5). The purified hCT2 protein was identified by western blotting (Fig. 3, lane 10).

Reconstitution of hCT2 in proteoliposomes and transport assay

To evaluate the hCT2 functionality and to perform a first biochemical characterization, the purified hCT2 was reconstituted in proteoliposomes and transport was assayed as described in Materials and Methods section. The reconstitution was carried out mimicking the procedure previously described for OCTN2 [25] due to the similarity of the substrate hypothetically transported by hCT2 and to the inclusion of hCT2 in the same cluster of OCTN-related proteins, as recently reported [26]. Indeed, using this strategy a hCT2-mediated carnitine transport could be detected in proteoliposomes, as function of time (Fig. 4). The hCT2 ability in mediating carnitine uptake was demonstrated by the much higher amount of taken up carnitine by proteoliposomes harboring the reconstituted hCT2 with respect to the diffusion observed in empty liposomes, i.e., liposomes without incorporated hCT2 (Fig. 4a). The hCT2 mediated transport, obtained by subtracting the controls from empty liposomes (Fig. 4b), followed a first order equation typical of a protein-mediated process with a calculated initial rate of 0.011 ± 0.0022 nmol/mg/min. Furthermore, the carnitine transport increased as a function of the protein amount incorporated into proteoliposomes, by an almost linear dependence (Fig. 4c). The transport reaction was unidirectional and sodium independent, as observed in Fig. 5a and in line with previously described data [6]. Finally, the dependence of the transport rate on carnitine concentration was studied (Fig. 5b). Data were fitted in a Michaelis-Menten equation of enzyme kinetics, that can be applied to transport processes as well [27], and a K_m of 288 ± 125 μ M and a V_{max} of 0.14 ± 0.03 nmol/mg/min were derived.

Conclusions

The described results represent, to our knowledge, the first report of heterologous expression of the human hCT2 in bacteria. This is the basis for performing characterization of this transporter that belongs to a SLC family whose structural characterization is still *in nuce*. Moreover, the over-expressed hCT2 is functionally active; this achievement, even though still preliminary, is a milestone for further structure/function relationships studies of this scarcely known transporter, that is most probably involved in crucial cell functions and is also linked to common human pathologies.

Declarations

Conflict of interest:

Michele Galluccio has no conflict of interest;

Tiziano Mazza has no conflict of interest;

Mariafrancesca Scalise has no conflict of interest,

Maria Chiara Sarubbi has no conflict of interest,

Cesare Indiveri has no conflict of interest.

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Figures

Figure 1

Effect of IPTG concentration on *E. coli* BL21 codon plus growth. BL21(DE3) codon plus cells transformed with hCT2-pH6EX3 construct were grown in absence (●) or in presence of 0.05 mM (●) or 0.1 mM (□), or 0.4 mM (△) IPTG, respectively. The cell growth was monitored for up to 8 hours after addition of the inducer. For measuring OD values, bacterial suspensions were diluted 1:4 and the absorbance values were adjusted according to the dilution factor. The values are means $\pm$ S.D. from three cell growths.

Figure 2

hCT2 expression in BL21 codon plus E. coli strain (a) E. coli BL21 codon plus strain was transformed with codon optimized hCT2-pH6EX3 (lanes 1-6) or hCT2-pCOLD I (lanes 7-9) construct. The protein synthesis was induced for six (lanes 1-3), eight (lanes 4-6) or twenty-two hours (lanes 7-9) in presence of 0.05 mM (lanes 1, 4, 7), 0.1 mM (lanes 2, 5, 8), or 1 mM IPTG (lanes 3, 6, 9). 35 μ g (total proteins) of the insoluble fractions of induced cell lysates were analyzed by western blotting analysis. (b) E. coli Lemo21

strain was transformed with hCT2-pH6EX3 (lane 1) or with codon optimized hCT2-pH6EX3 (lane 2). The protein synthesis was induced for 8 hours in presence of 0.05 mM IPTG. 35 μ g (total proteins) of the insoluble fractions of induced cell lysates were analyzed by western blotting analysis. (c) BL21 codon plus E. coli strain was transformed with hCT2-pH6EX3. The protein synthesis was induced for 8 h in the absence (positive control) or in the presence of different glucose and IPTG concentrations (lanes 2-7). Lane 1: pellet of the cell lysate induced (as in Fig. 2a lane 4) with 0.05 mM IPTG for 8 h in absence of glucose. Lanes 2-4: pellet of the cell lysate induced for 8 h in the presence of 0.5 % glucose and 0.05, 0.1 and 0.4 mM IPTG, respectively. Lanes 5-7: pellet of the cell lysate induced for 8 h in the presence of 1 % glucose and 0.05, 0.1 and 0.4 mM IPTG, respectively. 35 μ g (total proteins) were loaded in each lane.

Figure 3

Purification of the hCT2 protein. Proteins were separated by SDS-PAGE and stained as described in Materials and Methods. Lane 1: page ruler pre-stained plus marker; lane 2: pellet of the induced cell lysate; lane 3: pass-through fraction containing the unbound proteins; lane 4: fraction of the protein eluted with washing buffer added with 10 mM imidazole; lane 5: fourth fraction of the protein eluted with washing buffer added with 500 mM imidazole; lanes 6-10: western blotting analysis of the samples loaded as in 1-5.

Figure 4

[3H]carnitine Uptake in proteoliposomes. In (a), hCT2 was purified and reconstituted in proteoliposomes as described in Materials and Methods. Transport was started by adding 50 μ M [3H]carnitine to proteoliposomes (●) or control liposomes i.e., liposomes without incorporated protein (○). In (b), net protein mediated specific transport, obtained by subtracting uptake in control liposomes from uptake in proteoliposomes from Fig. 4a. Data were plotted according to first order rate equation. (C) dependence of transport on amount of reconstituted hCT2 in proteoliposomes. Transport was started by adding 50 μ M [3H]carnitine and stopped after 60 min. All results are means $\pm$ SD from three independent experiments. In (C), Multiple comparisons were performed using one-way ANOVA test followed by Tukey's test, p-value less than 0.01 was calculated. NS indicate non-significant difference between pair of groups estimated by Tukey's test.

Figure 5

Functional and kinetic characterization of hCT2 reconstituted in proteoliposomes. hCT2 was purified and reconstituted in proteoliposomes as described in Materials and Methods. In (a), effect of external sodium and internal carnitine on [3H]carnitine uptake in proteoliposomes. Transport was started by adding 50 μM [3H]carnitine and stopped after 60 min. Results are means ± SD from three independent experiments. No significant difference from the control was observed as estimated by Student's t-test ($P < 0.05$). In (b), kinetics of hCT2 reconstituted in proteoliposomes. Transport was measured in 15 min as uptake in proteoliposomes of [3H]carnitine at the indicated concentrations, as described in Materials and methods. Data was analyzed according to Michaelis-Menten equation as transport rate versus [3H]carnitine concentration. Results are means ± SD from three independent experiments.