Expression and characterization of a human pyruvate carboxylase variant by retroviral gene transfer

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Type A pyruvate carboxylase (PC) deficiency presents mainly in the Amerindian population, specifically the Ojibwa, Cree and Micmac tribes of the Algonquin-speaking peoples. The gene for PC contains a homozygous founder mutation (G¹⁸²⁸ → A) that results in an Ala⁶¹⁰ → Thr amino acid substitution in Ojibwa with Type A PC deficiency. The mutation is located in the highly conserved pyruvate-binding domain of PC. The present paper describes a retroviral expression system for human PC used to analyse the effects of this mutation. We show, through immunoblot analysis, PC enzyme activity assays, reverse-transcription PCR and mitochondrial-import experiments, that this mutation

is disease-causing in the Ojibwa population owing to its decreased catalytic activity, decreased steady-state levels of expression and inefficient import into the mitochondria. Our data suggest that this mutation may affect the stability of the protein, resulting in decreased steady-state levels of expression, and that it may also affect the secondary structure of the protein during the import process, thereby inhibiting proper translocation into the mitochondria, where PC is active.

Key words: Amerindian, mitochondrial import, pyruvate carboxylase deficiency, retroviral gene transfer.

INTRODUCTION

Pyruvate carboxylase (PC; EC 6.4.1.1) is a nuclear-encoded biotin-dependent mitochondrial enzyme that catalyses the ATP-driven formation of oxaloacetate from pyruvate and bicarbonate [1,2]. Oxaloacetate is the starting material for gluconeogenesis and is a substrate in other metabolic pathways. Therefore the PC-catalysed reaction is an important link between gluconeogenesis and other biochemical pathways. Besides gluconeogenesis, oxaloacetate is involved in the following: (1) the anaplerosis of tricarboxylic-acid-cycle intermediates [2–4]; (2) the transport of NADH/NAD⁺ in the malate–aspartate shuttle for the maintenance of redox equilibrium and ureagenesis [5–8]; (3) the glutamate–glutamine cycle for the *de novo* synthesis of neurotransmitters [9,10]; and (4) the transport of mitochondrial acetyl groups into the cytosol for fatty acid synthesis [11–13].

PC deficiency is an autosomal recessive trait affecting approx. 1 in 250 000 live births. PC deficiency ranges in severity from (1) the Benign (Type C) mild form causing only mild recurrent episodes of lacticacidosis with normal neurological development [14,15,16], through (2) the Simple (Type A) milder form in which the presenting features are lacticacidaemia and psychomotor retardation, to (3) the Severe (Type B) form in which neonatal lacticacidosis, hyperammonaemia and abnormal redox states result in death at less than 3 months of age [17–20]. To date, there have been four documented point mutations associated with Type A PC deficiency [21,22], two deletion mutations associated with Type B PC deficiency [23], and two point mutations associated with Type C PC deficiency (M. A. Carbone, S. Rahman, G. T. N. Besley, P. T. Clayton and B. H. Robinson, unpublished work). The exact mechanism by which these mutations function to elicit PC deficiency remains unexplained. In order to correlate known mutations with the spectrum of PC

deficiency, an expression system for studying mutant and wild-type alleles of PC is required.

While an expression and purification system in 293T kidney cells has been developed to obtain recombinant wild-type human PC [24], neither a bacterial expression system nor an expression system in PC-deficient human cell lines have been developed to study mutant forms of human PC. We report here a retroviral expression system in a human PC⁰ transformed skin fibroblast cell line. We compare expression of wild-type human PC with the Ala⁶¹⁰ → Thr (G¹⁸²⁸ → A) variant, the common Amerindian mutant associated with Type A PC deficiency in the Ojibwa population [21].

MATERIALS AND METHODS

Oligonucleotides for PCR reactions

All oligonucleotides were synthesized by the ACGT Corporation (Toronto, ON, Canada). Table 1 lists the sequences of oligonucleotides used in PCR reactions for these studies.

Table 1 Oligonucleotide sequences for PCR reaction primers

Underlined sequences refer to restriction sites as designated by the primer name.

Name	Target	Sense/antisense	Sequence (5'–3')
— 130 <i>Hind</i> III-F	PC	Sense	CTTACCGAATGAA/AGCTTGC
442-R	PC	Antisense	GAACCCGTAGCCAGGGTGCAC
3931 <i>Not</i> I-R	PC	Antisense	GC/GGCCGCCCTTCCCCAGGAGATAGGAC
pLNCX ₂ -F	pLNCX ₂	Sense	AGCTCGTTAGTGAACCGTCAGATC
pLNCX ₂ -R	pLNCX ₂	Antisense	ACCTACAGGTGGGGTCCTTCATTCCC
PGK-F	PGK	Sense	CAGTTTGGAGCTCCTGGAAG
PGK-R	PGK	Antisense	TGCAATCCAGGGTGACATG

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; ECL[®] (Amersham), enhanced chemiluminescence (of luminol); FCS, fetal-calf serum; α -MEM, α -modified Eagle's medium; PC, pyruvate carboxylase; PGK, phosphoglycerate kinase; RT, reverse transcription; SF, Superfect; SV40, simian virus 40; TBS-T, Tris-buffered saline/Tween.

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Mitochondrial-import studies

Mitochondria were isolated from rat hearts by the method of differential centrifugation as described by Cogswell and co-workers [25]. Mitochondria were resuspended to a final concentration of 3 mg/ml in import buffer [10 mM Hepes (pH 7.5)/2 mg/ml fatty-acid-free BSA/0.5 mM EGTA/70 mM sucrose/220 mM mannitol]. Full-length cDNAs encoding wild-type and $G^{1828} \rightarrow A$ PC variants were subcloned into the pcDNA3.1+ vector (Invitrogen) at the *HindIII* and *NotI* restriction sites, as described in the subsection entitled 'Construction of retroviral PC vectors'. The primers that were used to amplify PC cDNA were $-130HindIII$ -F and $3931NotI$ -R. Proteins were synthesized using the TNT T7 Coupled Reticulocyte Lysate System (Promega) using [^{35}S]methionine (Amersham) as the radioactive label. Mitochondria (120 μ g of protein), glutamate (33 mM) and rabbit reticulocyte lysates (5 μ l) were incubated with 2 μ l of lysate containing the translated radio-labelled precursor proteins at 30 °C for various time periods (10 min, 30 min and 1 h). The reactions were stopped by incubation on ice for 5 min. To visualize the reactions by SDS/PAGE, 5 μ l of each reaction mixture was combined with 20 μ l of 2 $\times$ SDS loading buffer. Samples were heat-denatured, and 5 μ l of each sample was electrophoresed through SDS/8%-(w/v)-PAGE gels. The gels were fixed, dried and exposed to X-ray film.

Transformation of PC-deficient cell lines

A skin fibroblast cell line from a patient with Type B PC deficiency and heterozygous for a TAGG deletion at the exon 15/intron 15 splice site and a GT deletion in exon 16 of the PC gene [23] was chosen as the target cell line for the subsequent expression studies. The cell line is devoid of functional PC mRNA and PC protein (cross-reacting-material-negative), as previously analysed by Robinson et al. [17–19]. This makes it an ideal candidate, since no endogenous PC is expressed. This 'PC⁰' cell line was transformed by transfecting the cell line with simian-virus-40 (SV40) large T antigen using a vector containing a large-T-antigen DNA. Transfections were carried out using Superfect (SF; Qiagen) according to the manufacturer's protocols, on cells 18–24 h after subculturing at a confluence of 60–80% in six-well (35 mm-diameter) plates. A portion (2 μ g) of column-purified (Qiagen) plasmid DNA was combined with 10 μ l of the SF reagent in 150 μ l of serum-free α -modified Eagle's medium (α -MEM)/2 $\times$ glucose (i.e. containing 10 mM glucose) medium and added dropwise to each of the six wells. Cells were subcultured 24 h after transfection at a ratio of one 35 mm-diameter plate per 83 mm-diameter plate. After approx. 2–3 weeks, transformed foci were visible and assayed for PC activity to confirm that there were no detectable changes in PC activity resulting from transformation. Western-blot analysis on mitochondrial fractions was also performed to confirm the absence of immunodetectable PC protein (results not shown). The transformed cell line is hereafter referred to as 'PC⁰-SV40'.

Retroviral gene expression of PC

Construction of retroviral PC vectors

Total RNA was isolated by the Trizol method (Invitrogen) from 83 mm-diameter plates containing 80–100% confluent skin fibroblasts. First-strand cDNA was synthesized using Superscript II (Invitrogen) and oligo(dT)₁₂ (ACGT Corporation). Wild-type PC cDNA was amplified from human liver first-strand cDNA using the primers $-130HindIII$ -F and $3931NotI$ -R. The mutant PC variant containing a $G^{1828} \rightarrow A$ mutation, which changes the

amino acid at position 610 from an alanine to a threonine residue, was amplified from a PC-deficient Type A Ojibwa patient's skin fibroblast RNA homozygous for the mutation.

The resulting cDNAs contained a *HindIII* and a *NotI* restriction-enzyme site, which were used for subcloning the amplicon into the pLNCX₂ retroviral vector (ClonTech) at the *HindIII* and *NotI* sites of the multiple cloning site. The resulting constructs were transformed to TOP10F' *Escherichia coli* cells. The plasmid DNA was isolated from culture medium (Luria–Bertani/ampicillin) after overnight growth at 37 °C from individual colonies using the Qiagen mini-prep spin kit (Qiagen). The wild-type PC clone is referred to as 'pLNCX₂(wt-PC)', and the $G^{1828} \rightarrow A$ mutant form as 'pLNCX₂($G^{1828} \rightarrow A$ -PC)'. These constructs, as well as the empty vector, 'pLNCX₂', were used in the subsequent transfection experiments.

Transfection of the packaging cell line

The viral packaging cell line PT67 was transfected as follows. On the day before transfection, one 83 mm-diameter plate of 80% confluent PT67 was subcultured to two six-well plates containing 3 ml of medium per well, and twelve 83 mm-diameter plates in 10 ml of medium. The PT67 cell line was grown in 'complete medium' [DMEM supplemented with 10% (v/v) fetal-calf serum (FCS), 2% (w/v) L-glutamine (200 mM), 1% penicillin/streptomycin and 1% sodium pyruvate (100 mM solution)]. The two six-well plates were used for transfections while the remaining twelve plates were allowed to reach 100% confluency for cryopreservation in 10% DMSO.

Transfection of the PT67 cell line was performed on the following day using SF reagent by following the manufacturer's protocol for stable transfection of adherent cells. Three wells were used per DNA construct. Each well contained one of the following DNA/SF ratios: 1:2 (5 μ g of DNA:10 μ l of SF), 1:5 (5 μ g of DNA:25 μ l of SF), 1:10 (5 μ g of DNA:50 μ l of SF) per DNA construct used. At 24 h following transfection, the PT67 cells were washed twice with PBS and replenished with 3 ml of complete medium. The cultures were incubated for 48 h to allow the viral titre to increase. After 48 h, each well was subcultured to an 83 mm-diameter plate in 10 ml of complete medium containing 300 μ g/ml G418 (geneticin) selection antibiotic. Cells were cultured in selection medium until large healthy colonies appeared. When the cells reached 80–100% confluency, they were subcultured 1:5 in complete medium lacking G418. The viral constructs were released into the complete medium. The viral-containing medium was collected and used to infect the target cell lines.

Infection of PC⁰-SV40 transformed skin fibroblast target cell lines

The target PC⁰-SV40 skin fibroblast cell line was plated 12–18 h before infection at a cell density of $(1-2) \times 10^5$ /60 mm-diameter plate using α -MEM/2 $\times$ glucose medium/10% FCS/1% penicillin/streptomycin medium. The target cell line was plated on four 60 mm-diameter plates so that each plate could be infected with one of the three constructs, while the fourth plate was left as the non-infected control.

On the day of infection, the medium from the packaging PT67 cells were collected using a 10 ml sterile syringe and filtered into a 50 ml sterile Falcon tube using a 0.45- μ m-mesh-size cellulose acetate sterile filter (Fisher; Mississauga, ON, Canada). The viral medium was diluted 2-fold using the α -MEM/2 $\times$ glucose medium/10% FCS/1% penicillin/streptomycin medium. Each plate of target cells was infected by adding 5 ml of this diluted viral medium. Polybrene (Sigma–Aldrich, Mississauga, ON,

Canada) was added to each infected plate to a final concentration of 8 µg/ml. After 5 h of incubation, the viral medium was replaced with fresh α -MEM/2 × glucose medium/10 % FCS/1 % penicillin/streptomycin (non-viral) medium. The cells were incubated for 48 h and then exposed to another round of infection.

At 24 h after the second round of infection, each of the 60 mm-diameter plates containing the infected cell lines were subcultured to 83 mm-diameter plates and incubated for 1 week in α -MEM/2 × glucose/10 % FCS/1 % penicillin/streptomycin medium containing 100 µg/ml G418 as the selective antibiotic. Following selection, the cells were allowed to grow in media without the G418 until large healthy colonies appeared. These colonies were isolated and analysed by immunoblotting, PCR and PC enzyme assay. The infection efficiency was determined by plating infected cells in parallel with and without selective antibiotic and comparing the number of colonies after the selection was complete. The infection efficiency was approximately equal (15–17 colonies) for each of the constructs.

SDS/PAGE and immunoblotting

Mitochondria were isolated from the PC⁰-SV40 retroviral transfected cell lines by the method of Pitkanen et al. [26]. Total protein concentrations were measured by the method of Lowry [27]. A portion (50 µg) of total protein was diluted to 35 µl in 2 × loading dye [10 % (v/v) glycine/80 mM SDS/62.5 mM Tris/HCl (pH 6.8)/5 % (v/v) 2-mercaptoethanol/0.2 % Coomassie Blue]. Samples were heat-denatured (95 °C) and electrophoresed through Tris/glycine/8 % polyacrylamide gels (Invitrogen) at a constant current of 25 mA. Gels were stained overnight in Coomassie Blue protein stain, destained for 2–4 h, fixed for 1–2 h in 30 % (v/v) methanol/3 % (v/v) glycerol and dried under vacuum at 80 °C for 1 h on Whatman® 3MM filter paper covered in SaranWrap (clingfilm). For immunoblotting, gels were transferred to nitrocellulose and blocked overnight at 4 °C in 5 % dried skimmed milk in Tris-buffered saline/Tween (TBS-T; 0.05 % Tween 20 in 20 mM Tris/HCl/150 mM NaCl, pH 7.5). The membrane was probed with rabbit anti-PC serum diluted 1:1200 in BSA/TBS-T for 2 h at room temperature. Anti-PC antibody was raised in rabbits against highly purified rat liver PC. It has good cross-reactivity with human PC [18]. Bound antibodies were visualized by the alkaline phosphatase/Nitro Blue Tetrazolium *p*-toluidine salt/5-bromo-4-chloroindol-3-yl phosphate ('NBT/BCIP') colorimetric method (Invitrogen) or by the horseradish peroxidase ECL® system (Amersham).

Quantitative reverse-transcription (RT) PCR analysis

Total RNA was isolated from the transfected skin fibroblasts using TRIzol® reagent (Invitrogen) and following the manufacturer's instructions. First-strand cDNA synthesis was carried out using total cellular RNA (20 µg) and oligonucleotide dT₁₂ with Superscript II reverse transcriptase (Invitrogen). The cDNA was PCR-amplified from first-strand cDNA using primers designed to detect exogenous PC mRNA as transcribed from the pLNCX₂ retroviral vector. The following primer pairs were used: pLNCX₂-F, with either pLNCX₂-R or 442-R. The internal control primers, PGK-F and PGK-R, were also included in some of the reactions. The primer pair pLNCX₂-F and pLNCX₂-R results in the full-length PC amplicon of size 4194 kb, whereas the pLNCX₂-F and 442-R primer pair results in an amplicon of size 629 bp. The internal control primers result in a product of 250 bp.

PC enzyme assay

The activity of PC in skin fibroblast cell lysates was based on a PC enzyme assay [28] modified from the method of Utter and Keech [11]. Briefly, skin fibroblasts were collected after trypsinization and washing. After resuspension in a small volume (1 mg of protein), 200 µl of 1 mM dithiothreitol and two rounds of freeze-thawing, PC was assayed by monitoring incorporation of ¹⁴CO₂ from H¹⁴CO₃ into oxaloacetate with pyruvate in the presence of acetyl-CoA and citrate synthase. After stopping the reaction with 0.2 M perchloric acid, samples were dried in a stream of nitrogen and the ¹⁴C incorporation measured after resolubilization in water by liquid-scintillation counting in Aquamix (ICN, Irvine, CA, U.S.A.). All reactions were performed in duplicate. The apparent *K_m* values for pyruvate of the mutant and wild-type retroviral PC were determined by performing the PC assay described and by varying the pyruvate concentration from 0 mM to 0.3 mM. The initial velocities were plotted against the various substrate concentrations and analysed by non-linear regression using GraphPad Prism®, version 1.03 (GraphPad Software Inc., San Diego, CA, U.S.A.).

For the dilution-inactivation of retroviral PC, the PC assays were performed as described above, using 0.3 mM pyruvate. Serial dilutions (0–6 mg/ml) of fresh skin-fibroblast whole-cell lysates were prepared and 10 µl of lysate was used per reaction.

RESULTS

The previous studies of the patient cell lines with the G¹⁸²⁸ → A mutation suggested that they had somewhat lower levels of PC protein, together with a major decrease in measurable PC activity.

Figure 1 Mitochondrial *in vitro* import of wild-type and Ala⁶¹⁰ → Thr PC proteins

Full-length precursor wild-type and Ala⁶¹⁰ → Thr ('Ala(610)Thr') PC proteins were synthesized from the pcDNA3.1 + T7 vector using the TnT-coupled reticulocyte-lysate system (Promega) with [³⁵S]methionine as the radioactive label. Samples were loaded and analysed by SDS/PAGE and exposure to X-ray film. (A) Import into isolated rat heart mitochondria was carried out for 10 min, 30 min and 1 h. (B) The supernatant (S, lanes 1 and 3) and the mitochondrial pellet (M, lanes 2 and 4) were loaded after a 3 h incubation period with the rat heart mitochondria.

Figure 2 Immunoblot analysis of isolated skin fibroblast mitochondria showing the retroviral expression of PC/pLNCX₂ proteins in PC⁰-SV40 transformed cell lines

Lanes 1–4, PC⁰-SV40 cell line transfected with no DNA (lane 1), pLNCX2 empty vector (lane 2), pLNCX2/G¹⁸²⁸ → A PC mutant (lane 3), and pLNCX2/wild-type PC construct (lane 4). Lanes 5–8, mitochondria from non-transformed skin fibroblast cell lines: lane 5, normal cell line; lane 6, Type A PC-deficient cell line; lane 7, Type C PC-deficient cell line; lane 8, Type B PC-deficient cell-line. Lane 9, isolated rat heart mitochondria. (A) ECL[®] immunoblot; (B) Horseradish peroxidase visualization of the immunoreactive protein for lanes 3 and 4.

To investigate this, we carried out investigations of mitochondrial import and retroviral expression for wild-type and G¹⁸²⁸ → A PC.

Mitochondrial import

To investigate whether decreased immunoreactive PC protein of the G¹⁸²⁸ → A PC variant compared with wild-type PC was caused by deficient mitochondrial import, [³⁵S]methionine-labelled G¹⁸²⁸ → A PC, and wild-type PC proteins were synthesized *in vitro* and then incubated with freshly prepared rat heart mitochondria. The amounts of wild-type and mutant PC that were imported into the mitochondria were compared. Densitometric analysis revealed that the wild-type PC was imported after at least 10 min, where the mutant was not, even after 1 h of incubation (Figure 1). This could imply that the Ala⁶¹⁰ → Thr substitution resulting from the G¹⁸²⁸ → A point mutation may affect translocation of the protein across the mitochondrial membrane, owing to differences in secondary structure compared with wild-type PC.

Retroviral expression of wild-type and G¹⁸²⁸ → A PC

In order to investigate the expression of the mutant enzyme, both wild-type and G¹⁸²⁸ → A PC protein were expressed in an SV40-transformed cell line with zero PC activity (PC⁰-SV40) due to null mutations [23].

Mitochondria were isolated from the PC⁰-SV40-transformed cell lines, and levels of expression of PC protein were compared with those in non-transformed cell lines from normal, Type A, B and C PC-deficient cell lines. The immunoblot is shown in Figure 2(A). Densitometric analysis of the PC band revealed that the steady-state levels of wild-type PC expressed from the retroviral vector is present in more than twice the concentration compared

Figure 3 Quantitative RT-PCR analysis of retroviral PC expression in PC⁰-SV40-transformed cell lines

The PGK/PC ratio was measured by densitometry and was calculated to be 2:1 after 24 cycles for both the G¹⁸²⁸ → A ('1828G A') and wild-type (WT) PC. Lane M is the incremental ladder (123 bp, 246 bp, 369 bp ...). All other lanes are as indicated on the Figure.

Table 2 PC activities from retroviral expression of human recombinant PC

The numbers of replicate measurements are shown in parentheses.

Skin fibroblast cell line	PC activity (nmol/min per mg of protein)
Non-transformed controls	0.41 ± 0.25 (6)
PC ⁰	0.010 ± 0.005 (5)
PC ⁰ SV40	0.010 ± 0.004 (2)
PC ⁰ SV40-pLNCX ₂	0.006 ± 0.004 (2)
PC ⁰ SV40-pLNCX ₂ (wild-type PC)	3.84 ± 0.75 (5)
PC ⁰ SV40-pLNCX ₂ (G ¹⁸²⁸ → A PC)	0.35 ± 0.19 (3)

with the G¹⁸²⁸ → A mutant counterpart. When compared with the PC expressed in the normal skin fibroblast cell line shown in lane 5, the expression of the mutant is approximately equal to it, while that of the wild-type expressed at more than twice its steady-state level. The overexpression is due to the presence of the long terminal repeats, which drives high-level constitutive expression of the target gene in mammalian cell lines. The colorimetric immunoblot shown in Figure 2(B) revealed the presence of protein degradation products of the mutant PC. It is possible that the decreased steady-state level of mutant PC in the Type A PC deficiency Amerindian patients is a result of the protein's decreased half-life relative to the wild-type.

To determine whether the decreased level of mutant PC protein is a result of decreased mRNA concentration, quantitative RT-PCR analysis was carried out on the RNA of the expressed constructs. The results are shown in Figure 3. Phosphoglycerate kinase (PGK) was included as the internal control. Densitometric analysis revealed that the transcription of the wild-type and G¹⁸²⁸ → A gene occurs at approximately equivalent rates. Therefore the decreased titre of mutant PC protein is not a result of decreased transcription of the mutant relative to wild-type. The PC activity on total cell lysates from the transfections was measured. The results are shown on Table 2. The activity of the mutant PC protein is 9% of that of the wild-type PC protein.

Kinetic studies of wild-type and Ala⁶¹⁰ → Thr retroviral PC

Table 3 shows the apparent $V_{\max}$ and $K_{m,\text{pyruvate}}$ values which were derived from the plots shown in Figure 4. The apparent $K_{m,\text{pyruvate}}$ values for the control and the retroviral wild-type PC were similar (≈ 0.1 mM), while that for the Ala⁶¹⁰ → Thr retroviral mutant PC was slightly decreased (≈ 0.08 mM). The apparent $V_{\max}$ values for the three samples were significantly

Table 3 Apparent $V_{\max}$ and $K_{m,\text{pyruvate}}$ values for mutant and wild-type retroviral expressed PCResults are means $\pm$ S.D. ($n = 3$).

Cell lysate	$V_{\max}$ (nmol/min per mg)	$K_{m,\text{pyruvate}}$ (mM)
Ala ⁶¹⁰ → Thr cell line	0.06 $\pm$ 0.02	N.D.*
Control cell line	1.17 $\pm$ 0.10	0.11 $\pm$ 0.02
Ala ⁶¹⁰ → Thr retroviral PC	4.86 $\pm$ 0.10	0.08 $\pm$ 0.00
Wild-type retroviral PC	8.48 $\pm$ 0.52	0.12 $\pm$ 0.02

* Not determined.

Figure 4 Apparent K_m for pyruvate

The PC assays were carried out radioactively (using [¹⁴C]bicarbonate) with pyruvate concentrations as indicated and 60 mg of fresh skin fibroblast whole-cell lysate per reaction. All other components (ATP, acetyl-CoA, Mg²⁺ and KHCO₃) were present at concentrations at least 3-fold that of their K_m values for chicken liver PC [34]. Shown are the plots for a control skin fibroblast cell line (□), Type A PC-deficient skin fibroblast cell-line homozygous for the Ala⁶¹⁰ → Thr [Ala(610)Thr] mutation (△), wild-type retroviral PC (■) and Ala(610)Thr mutant retroviral PC (▲). The values for $V_{\max}$ and $K_{m,\text{pyruvate}}$ were calculated using GraphPad Prism®, version 1.03, and are listed in Table 3.

different and may be attributed to the differences, at least partially, between steady-state levels of mitochondrial PC within each cell lysate sample. The mitochondrial Western blot in Figure 2 demonstrates that the retroviral wild-type PC shows levels of expression that are double that of the retroviral mutant PC and 2.5 times higher than the control cell line. Note that Figure 2 is a representative blot which shows that in a Type A PC-deficient patient, the steady-state level of PC is expressed typically at 45 % the level of the control cell line shown in lane 5. This is comparable with the differences in steady-state levels shown by the retrovirally expressed proteins. Densitometric analysis of [³H]biotin-labelled PC proteins [17–19] revealed that steady-state levels of Type A PC-deficient patients ranges from 4 to 50 % relative to control cell lines, with an average steady-state level of 28 % ($n = 10$). The PC activity of Type A PC-deficient patients averages 0.0624 ± 0.0211 nmol/min per mg ($n = 10$) compared with control values of 1.217 ± 0.3772 ($n = 18$). It has been observed that control values for PC activity can vary anywhere between 0.3 and 3.5 nmol/min per mg of protein, depending on the cell line and subculture number. Table 4

Table 4 Estimation of the decrease in specific activity by comparison of steady-state levels and PC activity between retroviral and endogenous PC proteins

The ratio PC/steady-state in the last column was calculated as follows:

(PC activity_{wt}/steady-state levels_{wt})/(PC activity_{Type A}/steady-state levels_{Type A})

The subscript_{wt} refers to either control cell lines expressing endogenous wild-type PC or cell lines expressing wild-type Retroviral PC. Type_A refers either to cell-lines of Type A PC-deficient patients homozygous for the Ala⁶¹⁰ → Thr mutation or to cell lines expressing the Ala⁶¹⁰ → Thr retroviral PC.

Cell type	Steady-state levels (by densitometry) (%)	PC activity (nmol/min per mg)	PC/steady state
Control (wild-type PC)	100	1.22 $\pm$ 0.37 ($n = 18$)	
Type A PC-deficient	28	0.06 $\pm$ 0.02 ($n = 10$)	5.46
Wild-type retroviral PC	256	3.84 $\pm$ 0.75 ($n = 5$)	
Ala ⁶¹⁰ → Thr retroviral PC	132	0.35 $\pm$ 0.19 ($n = 3$)	5.75

compares the ratio of wild-type to mutant PC in terms of PC activity and steady-state levels. In an attempt to normalize values to specific activity, the ratio of 5.46 (endogenously expressed PC) was calculated and found to be comparable with the value calculated for the retrovirally expressed PC (5.75). The similar ratios imply that the retroviral expression system is a good indicator of the situation occurring in control and Type A PC-deficient cell lines.

To examine the PC activity of retroviral PC at various lysate concentrations, serial dilutions of wild-type and mutant retroviral PC lysates were prepared and assayed as outlined above. Figure 4 shows the plots of the wild-type and mutant retroviral PC over a range of concentrations. For the wild-type PC, optimal PC activity occurred between 0.25 and 0.50 mg/ml of lysate, while for the mutant, optimal activity occurred between 1.0 and 2.0 mg/ml of lysate. In both cases, PC activity increased to a maximum as lysate concentration decreased, and then decreased to negligible values below their respective optimal lysate concentrations.

DISCUSSION

The present work demonstrates the recombinant expression of human PC by retroviral gene transfer and the subsequent analysis of a mutant PC variant common in the Ojibwa population [21].

To examine whether decreased levels of mutant protein are due to diminished import into the mitochondria, the import efficiency of the mutant and wild-type PC into intact rat heart mitochondria was investigated. The import efficiency of mutant compared with wild-type PC is impaired, as shown on Figure 1. While the wild-type is able to translocate into the mitochondria within 10 min, the mutant appears unable to do so efficiently after 1 h (Figure 1A). After a 3 h incubation period (Figure 1B), only 50 % of the mutant could be translocated across the mitochondrial membrane. This could imply that the Ala⁶¹⁰ → Thr replacement resulting from the G¹⁸²⁸ → A point mutation may alter the translocation of the protein across the mitochondrial membranes, owing to differences in secondary structure compared with wild-type PC. However, the presence of a protein of correct molecular mass in mitochondria from the patients and PC⁰ cell lines transfected with the Amerindian mutant suggests that a certain level of translocation is possible. The decreased levels of PC protein of G¹⁸²⁸ → A mutant cell mitochondria may be due, at least in part, to a deficiency in import of the mutant protein.

Figure 5 Secondary-structure prediction of human PC

(A) Predicted secondary structure of wild-type PC. (B) Predicted secondary structure of Ala⁶¹⁰ → Thr PC mutant. The analysis was performed using the PredictProtein Server [35] (<http://cubic.bioc.columbia.edu/predictprotein/>). Shown is the amino acid sequence of residues 600–660 of human PC (top line) and the predicted secondary structure motifs (PHD_sec). In the bottom line in each case: H, helix; E, extended sheets (E); blank, loops/other.

Recombinant human PC was expressed in a PC⁰ cell line by retroviral gene transfer. Complementation of the defect after viral expression led to reconstitution of PC activity at a level higher than usually seen in wild-type control cells. Immunoblot analysis on mitochondrial extracts demonstrated that the recombinant PC protein was, at least in part, properly transported to the mitochondria where PC is active, since activity and protein were both readily detected (Figure 2). This expression system was used to characterize the common mis-sense mutation (G¹⁸²⁸ → A) previously described in an Ojibway–Cree population with Type A PC deficiency [21]. This mutation, when expressed in skin fibroblasts under the same conditions as wild-type PC, had a PC activity 9 % of the wild-type (see Table 2). Immunoblot analysis on mitochondrial extracts revealed that the steady-state level of the mutant protein was 50 % of that of the wild-type (see Figure 2, lanes 3 and 4). In addition, the colorimetric immunoblot (Figure 2B) shows evidence of degradation protein products resulting from the mutant PC protein. These protein bands detected by the PC antibody, because they are in mitochondrial extracts, suggests some intramitochondrial degradation. It is possible that the decreased steady-state level of mutant PC protein seen in the Type A PC deficiency Amerindian patients is a result partly of the protein's decreased half-life relative to the wild-type. Protein folding and proteolysis are known to be closely linked processes [29,30]. The decay of mutant PC polypeptides protects the mitochondria against accumulation of misfolded, and possibly toxic, proteins. This may ultimately protect Type A PC-deficient patients from the more severe consequences such as those exhibited by Type B PC-deficient patients.

To examine whether decreased steady-state levels of mutant PC protein are a result of decreased steady-state levels of mRNA, quantitative RT-PCR analysis was carried out on the mRNA extracted from the skin fibroblasts containing the viral PC constructs. As shown in Figure 3, the rates of transcription between wild-type and mutant are equivalent when normalized against the internal control standard. Thus the decreased steady-state level of protein expression of the mutant is not a result of decreased mRNA concentration.

The kinetic evaluation of the retroviral expressed wild-type and Ala⁶¹⁰ → Thr PC proteins revealed that their $K_{m,\text{pyruvate}}$ values were similar, but their V_{max} rates differed. The differences

in V_{max} observed in the expression system can be explained by the different steady-state levels of wild-type compared with the mutant in the mitochondria where PC is active. The similar K_{m} values implies that the Ala⁶¹⁰ → Thr mutation does not affect pyruvate binding during the catalytic reaction. According to secondary-structural predictions of wild-type and mutant PC (Figure 5), the Ala⁶¹⁰ residue is located upstream of a helical region (Figure 5A). The substitution of threonine for the alanine residue does not alter the predicted secondary structure (Figure 5B). The data indicate that the Ala⁶¹⁰ → Thr mutation affects the import of PC into the mitochondria and the stability of PC protein, but not its binding affinity for pyruvate. The $K_{m,\text{pyruvate}}$ value for the Type A PC-deficient cell line homozygous for the Ala⁶¹⁰ → Thr mutation could not be reliably calculated, because the enzyme concentration and PC activity are too low. There seems, however, to be a discrepancy between the apparent amount of PC protein and the measurable PC activity, i.e. the mutant when over-expressed had seemingly better activity relative to control cell lines. This requires some explanation.

A recent study by Attwood and Geeves [31] showed evidence of the phenomenon known as 'dilution inactivation' in which the specific activity of chicken liver PC was decreased as the enzyme concentration was decreased. The inactivation of PC has been shown to be associated with the dissociation of the active tetrameric form of the enzyme into inactive dimers and monomers [32,33]. The low activity of the naturally occurring mutant PC compared with its level of expression could very well be due to such a phenomenon. We examined this possibility using retroviral wild-type and mutant PC, by preparing serial dilutions of each lysate and measuring their PC activity (Figure 6). The retroviral wild-type PC reached a maximum PC activity (5.67–5.72 nmol/min per mg) when lysates were present in concentrations of 0.5–1.0 mg/ml, which is approx. 67 % of the apparent V_{max} (Table 3). For the mutant, maximum activity (1.80–1.88 nmol/min per mg) occurred at lysate concentrations between 1.0 and 2.0 mg/ml, which is approx. 37 % of the apparent V_{max} (Table 3). It should be recalled from Figure 2 that the steady-state levels of PC protein of the wild-type retrovirally expressed PC is double that of the mutant. The insert to Figure 6 has been adjusted to correct for the concentration of PC relative to wild-type and results in an optimal activity that is 75 % of the apparent V_{max} for the mutant. Below their optimal concen-

Figure 6 Dilution inactivation of retroviral PC

The PC assays were carried out using radioactive [^{14}C]bicarbonate and a pyruvate concentration of 0.3 mM. Serial dilutions of fresh skin-fibroblast whole-cell lysates were prepared. Their protein concentrations are indicated on the abscissae. All other components (ATP, acetyl-CoA, Mg^{2+} and KHCO_3) were present at concentrations at least 3-fold that of their K_m values for chicken liver PC [34]. Shown are the plots for wild-type retroviral PC (■) and Ala⁶¹⁰ → Thr mutant retroviral PC (▼). The insert accounts for differences in steady-state levels between wild-type and Ala⁶¹⁰ → Thr mutant retroviral PC.

trations, the PC activities decreased to a minimum, possibly indicating the dissociation of active homotetramers to inactive monomers and dimers. Above their optimal concentrations, their PC activities decreased to steady-state levels with increasing lysate concentrations. Increases in concentrations possibly lead to the formation of aggregates of PC homotetramers, leading to a decrease in activity.

One possible mechanism to explain the role that the Ala⁶¹⁰ → Thr mutation plays in Type A PC deficiency is that the mutation induces some local protein instability resulting in decreased steady-state levels of expression and decreased mitochondrial import. The decreased concentration of mutant protein in the mitochondria, likely results in dilution inactivation. Since one of the main roles of PC is to replenish the pools of metabolic intermediates of the tricarboxylic acid cycle, low levels of active PC would result in the decreased ability of PC to sustain the tricarboxylic acid cycle. This ultimately results in the symptoms displayed by the Ojibwa–Cree population with Type A PC deficiency, including, lactic acidemia and psychomotor retardation. In their study of dilution inactivation, Attwood and Geeves [31] demonstrated the re-activation of dilution-inactivated PC enzyme upon incubation with ATP, MgCl_2 and acetyl-CoA. Their data may provide a clue for developing treatment strategies for patients with Type A PC deficiency. Alternatively, if a method could be developed for driving increased expression of the mutant PC, a more healthy level of PC activity would be seen in these patients.

Ala⁶¹⁰ is evolutionary conserved between bacteria and humans and lies within a sequence of high identity, thereby implicating this residue as a significant one for either catalysis or structural integrity of PC. It is also possible that Ala⁶¹⁰ is involved in the formation of active PC tetramers, and an amino acid substitution may result in altered homotetramer formation. The data presented here suggests a correlation between G¹⁸²⁸ → A (Ala → Thr) mutation and Type A PC deficiency in the Ojibwa–Cree

population. We have demonstrated the reconstitution of PC transcription, translation and activity in a transformed cell line devoid of functional PC mRNA and protein. It was shown that the PC is targeted and expressed in the mitochondria, where PC is active. Further experiments, including X-ray crystallography and more detailed kinetic studies, will help to conclusively evaluate the structure–function relationship of important amino acid residues of PC. Using the retroviral expression system, other PC mutants can now be evaluated. To date, there is no effective treatment for those afflicted with PC deficiency. However, this strategy may be used to develop a method of gene therapy for these patients.

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