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Expression of human pyruvate carboxylase in insect cells using the baculovirus system

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Abstract

Constructs containing cDNA encoding human pyruvate carboxylase (PC) with and without a hexahistidine (6× His) tag at the N-terminal of the mature enzyme have been cloned under the control of the polyhedrin promoter. These two constructs were co-transfected with the baculovirus genome into Sf9 cells to produce recombinant baculoviruses harbouring human PC cDNA. The expression of human PC under the control of the polyhedrin promoter was found to be at its highest level at 4 days post-infection. The expressed material accounted for up to 70% of total cellular protein with 5% of this expressed material being found in the soluble fraction. The recombinant human 6× His-PC isolated with a purity of ~50% using a Ni–NTA agarose column was found to have the specific activity of 7 U/mg, which was similar to that produced from a 293T stable line [Biochem. Biophys. Res. Commun. 266 (1999) 512]. This is the first report of a heterologous expression system for recombinant human PC.

Keywords: Pyruvate carboxylase; Gluconeogenesis; Biotin containing enzyme; Baculovirus expression system

Pyruvate carboxylase (PC) is a biotin enzyme that catalyzes the ATP-dependent carboxylation of pyruvate to oxaloacetate. Therefore, this enzyme plays an anaplerotic role in many tissues including gluconeogenesis in liver and kidney, lipogenesis in adipose tissue, and glucose-induced insulin release from pancreatic β-cells [1]. In rat, PC expression is subjected to both transcriptional and translational regulation [2]. Two tissue-specific promoters are responsible for alternative transcripts of the PC gene under different physiological stimuli [2,3]. In human liver and kidney two alternate transcripts bearing different 5′-untranslated regions have also been reported [4] and are likely to be produced from two distinct promoters as in the rat [4,5].

PC deficiency in humans causes symptoms ranging from mild lactic acidosis to severe lactic acidosis with mental retardation depending on the type of mutations. The milder form is associated with point mutations in the coding exons [5,6] while the severe form is associated with intron retention and frameshift mutations [7].

With the increase in case reports of PC mutations in humans, expression systems for recombinant human PC have been established with the aim of characterizing mutant enzymes that were identified in patients. A homologous expression system in the human embryonic kidney cell line 293T with an endogenous level of PC [8], and a retroviral expression system in a PC deficient cell line [9] have been reported. Here we report a heterologous expression system for producing human PC in insect cells using the baculovirus expression system in serum-free medium. The recombinant material produced by this method was found in both soluble and insoluble fractions. Furthermore, human PC with a hexahistidine tag at the N-terminal of the mature enzyme was engineered to facilitate the purification procedure.

Materials and methods

Generation of expression constructs. The full-length human PC cDNA without histidine tag was excised from the pEF-PC plasmid [8]

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with *Eco*RI and *Bam*HI, and ligated to *Eco*RI–*Bam*HI digested pAcSG His NT-A vector [Pharmingen, USA (kindly provided by Professor C.-F. Lo, Department of Zoology, National Taiwan University, Taipei)]. The human PC with the N-terminal hexahistidine tag was engineered as follows. Briefly, the pPCR800-11 clone containing 5′-end of human PC cDNA [8] was used as the template to insert a DNA sequence encoding hexahistidine after the mitochondrial targeting sequence using the Quick change site-directed mutagenesis kit (Stratagene, CA, USA). Briefly, the mutagenesis was performed in a 50 μl reaction mixture containing 1× *Pfu* polymerase buffer (100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris–HCl, pH 8.8, 20 mM $MgSO_4$, 1% (v/v) Triton X-100, and 1 mg/ml bovine serum albumin), 120 ng ForHis (5′-gtccggcgcctggagtataag**caccaccaccaccaccat**ggccccatcaagaaa gtcatggtg-3′; bold represents the nucleotide sequence encoding hexahistidine), and 120 ng RevHis (5′-caccatgactttcttgatggggcc**atggtggtg gtggtggtg**cttatactccaggcgccggac-3′; bold represents the nucleotide sequence encoding hexahistidine), 2.5 mM of each dNTP, and 2.5 U *Pfu* polymerase (Stratagene). The PCR profile consisted of an initial denaturation at 95 °C for 30 s followed by 20 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min, and extension at 68 °C for 8 min. The template DNA was digested with *Dpn*I overnight before being transformed into DH5α. The presence of the DNA sequence encoding hexahistidine was verified by DNA sequencing. This 5′-end clone was then ligated to the 3′-end of human PC cDNA as previously described [8]. The 3.6 kb *Bam*HI–*Eco*RI fragment containing full-length human PC cDNA was ligated to the *Bam*HI–*Eco*RI digested pAcSG His NT-A vector.

Generation of recombinant baculoviruses harbouring hPC and 6× His-hPC. To produce recombinant baculoviruses by homologous recombination, the recombinant pAcSG His NTA plasmids were co-transfected into Sf9 (Gibco, USA) with linearized baculovirus genome. Briefly, Sf9 cells exponentially grown in SF900 II serum-free medium (Gibco, USA) were seeded at a density of 7.5×10^5 cells/35 mm dish. The cells were washed with the antibiotic-free medium twice. Recombinant pAcSG plasmid (300 ng) and 25 ng of triple cut baculovirus DNA (BacVector 10000, Novagen) were complexed with 1 μg of Eufectin reagent before being transfected into Sf9 cells. After 1 h of transfection, 1.2 ml of complete medium was added and the transfected cells were maintained at 27 °C. At 7 days post-transfection, the culture medium containing the recombinant baculoviruses was collected from the dish and diluted 10^4-, 10^5-, and 10^6-fold. The recombinant baculoviruses were further screened by end point limiting dilution and nucleic acid hybridization as described by Tsai et al. [10]. The primary viral stock obtained by this procedure was amplified to $>10^7$ pfu/ml and kept as the working stock.

Recombinant production of hPC and 6× His-hPC in Sf9 by baculoviruses. Cells (6×10^6) were seeded in a 55 cm^2 culture dish and grown in 10 ml SF900 medium, and then infected with recombinant baculoviruses with a multiplicity of infection (MOI) of 0.1. The infected cells were maintained at 27 °C for 96 h before being harvested. Whole cell lysates were prepared by adding 2× SDS loading dye directly to the cell pellet. The soluble protein fraction was prepared as follows: routinely 30×55 cm^2 culture dishes containing infected Sf9 were used in this experiment. At 4 days post-infection, the culture medium was aspirated and the cells were scraped from the flasks and centrifuged at 1200*g* for 2 min. The cell pellet was washed once with 0.1 M phosphate buffer, pH 7.4, resuspended in 36 ml lysis buffer [50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 1 mM PMSF, and 1% (v/v) Nonidet P40, pH 8.0] and left on ice for 20 min. The lysate was centrifuged at 13,000 rpm for 5 min at 4 °C. The supernatant and pellet were analyzed by SDS–PAGE and Western blotting.

Purification of 6× hPC using Ni–NTA column. The supernatant containing the soluble 6× His-hPC was incubated with 1.5 ml of 50% (v/v) Ni–NTA agarose [Qiagen] (equilibrated in lysis buffer) at 4 °C with gentle rocking for 1 h. The mixture was then loaded on a 10 ml column under gravity flow. The column was washed twice with 3 ml wash buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, and 20 mM imidazole). The 6× His-hPC was eluted from the column with 4×0.75 ml elution buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, and 250 mM imidazole). The eluted fractions were pooled and precipitated by adding 100% saturated ammonium sulfate solution, pH 7.0, to a final concentration of 40% saturation and stirring at 4 °C for 30 min, and then centrifuged at 20,000*g* for 30 min. The pellet was dissolved in 100 μl buffer A (25 mM potassium phosphate buffer, pH 7.8, 40 mM ammonium sulfate, and 0.1 mM DTT).

SDS–PAGE, Western blot analysis, and PC activity assay. Proteins were separated by 7.5% discontinuous SDS–PAGE and subjected to Western blotting as previously described [8]. The blots were reacted with anti-6× His monoclonal antibody (Amersham–Pharmacia) followed by adding anti mouse IgG polyclonal antibody conjugated with alkaline phosphatase (Zymed, USA) or with avidin conjugated with alkaline phosphatase (Sigma). PC activity assay was performed with 10 μg of partially purified 6× His-hPC using the assay conditions described previously [8].

Results and discussion

We have engineered the N-terminal of human PC to include a hexahistidine tag to facilitate the purification procedure. We have inserted the hexahistidine at the N-terminal rather than the C-terminal as it has previously been shown that the extension of the C-terminal portion of the yeast PC2 isozyme by a few amino acid residues resulted in poorer biotinylation [11]. Since the cleavage site of the N-terminal mitochondrial targeting sequence was predicted to occur between Arg31 and Leu32 [12], we inserted 6× histidines and an extra glycine between Lys35 and Pro36 (see Fig. 1). This was intended to minimize disruption of the protease recognition site that may result in the failure of PC to be targeted to the mitochondrial matrix. We initially examined this possibility by transfecting this construct cloned in the mammalian expression vector, pEFIRES-Puro [13], into 293T cells and found that this 6× His-hPC was successfully targeted to the mitochondria (data not shown). The cDNA encoding this 6× His-hPC was then subcloned into the baculovirus transfer vector which was then co-transfected with the baculovirus genome. After the selection procedure, 5 out of 14 selected recombinant viral clones were found to express human PC with different expression levels as shown in Fig. 2A. The size of human PC produced by the recombinant baculoviruses agreed with that produced by 293T [8], i.e., ~116 kDa. As post-translationally modified PC contains a single biotin moiety attached to a specific lysine at the C-terminal by biotin protein ligase [14], this allowed us to detect the biotinylated PC using avidin-alkaline phosphatase. As seen in Fig. 2B, the 116 kDa overexpressed band seen on the Coomassie stained gel reacted with avidin, suggesting that the recombinant hPC produced by Sf9 was correctly folded as it was readily recognized and biotinylated by biotin protein ligase. The upper band (~130 kDa) that also reacted with avidin is the endogenous PC produced by host [15]. Clone D7 was selected and amplified to a high titer for

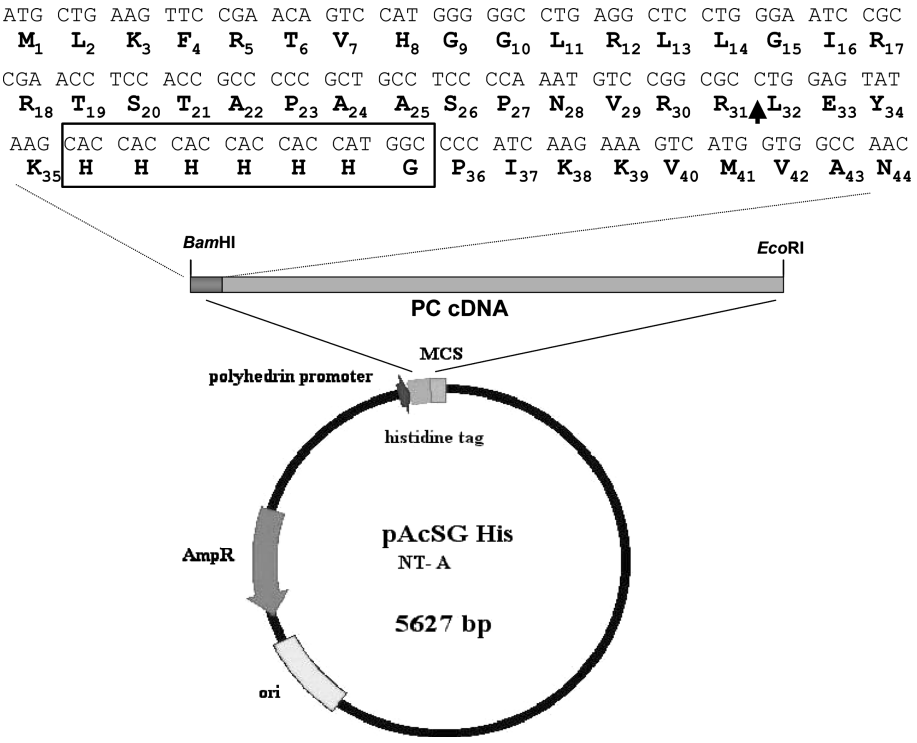

Fig. 1. Schematic diagram showing the baculovirus transfer vector pAcSG His NT-A containing 6× His-hPC cDNA inserted at the multiple cloning site (MCS). Also shown is the N-terminal mitochondrial targeting sequence with six histidines inserted (boxed). Arrow represents the predicted protease cleavage site between R31 and L32 [12].

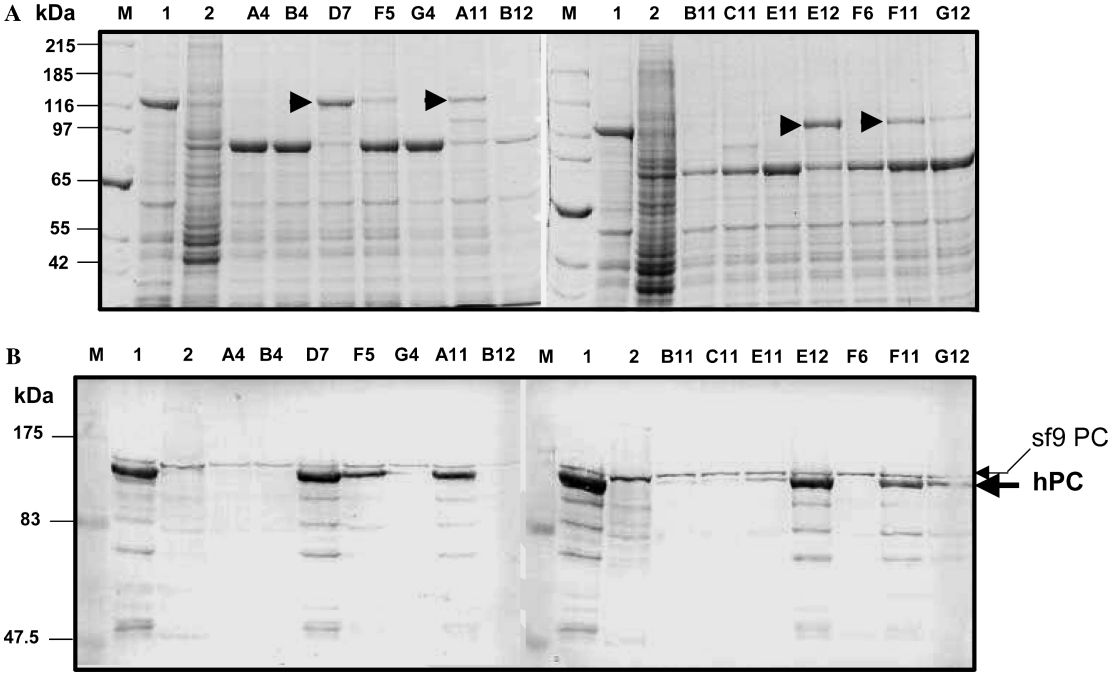

Fig. 2. Screening of different putative recombinant baculoviral clones harbouring 6× His-hPC. Sf9 were infected with indicated recombinant baculoviral clones for 96 h. Whole cell lysates were prepared, analyzed by SDS–PAGE, stained with Coomassie (A), and subjected to Western blotting using avidin-conjugated alkaline phosphatase (B). Thick arrows represent bands that correspond to 6× His-hPC (116 kDa) while the thin arrow represents Sf9 PC. M, protein markers; 1, Sf9 infected with baculovirus harbouring untagged hPC; 2, non-infected Sf9.

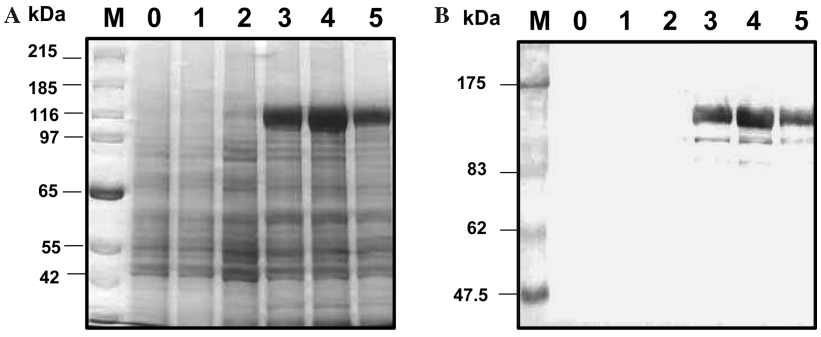

Fig. 3. Time course expression of 6× His-hPC in Sf9 infected with baculovirus D7 clone. Sf9 cells were infected with baculovirus D7 clone. At 0, 24, 48, 72, 96, and 120 h post-infection (lanes 0–5, respectively), the infected cells were collected and whole cell lysates were prepared, analyzed by SDS–PAGE, and stained with Coomassie blue (A) or subjected to Western blot with 6× His monoclonal antibody (B). M, protein markers.

further studies. The time course induction of human PC produced in Sf9 was studied by harvesting infected cells at different times post-infection. As shown in Fig. 3A, the recombinant 6× His-hPC was undetectable at 24 and 48 h post-infection, but its expression was rapidly enhanced at 72 h post-infection and was maximized at 96 h post-infection. However, the expression level was decreased by 120 h post-infection. Western analysis using anti-6× His monoclonal antibody reacted strongly with recombinant 6× His-hPC, confirming that the N-terminal 6× His was not cleaved off during targeting to the mitochondrial matrix (Fig. 3B). Densitometer scanning of the 116 kDa band observed on Coomassie-stained gel shows that the expressed hPC accounted up to 70% of total protein at 96 h post-infection. The late expression of hPC in Sf9 is consistent with the late expression of polyhedrin promoter [16,17] which was used to regulate hPC expression. Overexpression of many transgenes regulated by the polyhedrin promoter often leads to the aggregation of protein as the result of robust transcription/translation in combination with the limited amounts of chaperones which assist folding of proteins in the cells. To determine the proportion of the insoluble and the soluble material of hPC produced in Sf9, we fractionated the soluble and insoluble material by treating the cells with the non-ionic detergent, i.e., NP40. As shown in Fig. 4A, less than 10% of the totally expressed material (pellet plus soluble) were found in the soluble fraction whilst the rest of material was found in the insoluble fraction. The 116 kDa band was enriched after subjecting the soluble fraction to Ni–NTA chromatography (Fig. 4A). The eluted fractions (E1–E4) were pooled and precipitated with 40% saturated ammonium sulfate solution. The purity of 6× His-hPC after ammonium sulfate precipitation was approximately 50% pure as judged by SDS–PAGE. The final yield of partially purified hPC after ammonium sulfate precipitation of eluted fractions was about 60 μg started from 1 ml packed volume of infected Sf9. An activity assay of partially purified 6× His-hPC yielded a specific activity of 7 U/mg, similar to that of the hPC partially purified from mitochondria of

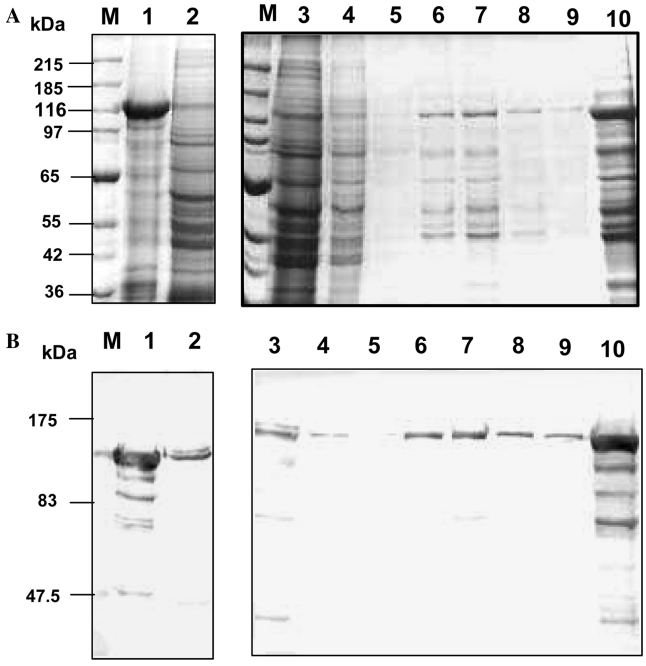

Fig. 4. SDS–PAGE and Western blotting of 6× His-hPC purified from Ni–NTA column. Sf9 cells were infected with recombinant baculovirus D7 for 96 h. One milliliter of packed cells was harvested and the soluble fraction was isolated from insoluble fraction by centrifugation. The soluble fraction was subjected to Ni–NTA chromatography. 6× His-hPC was eluted from column with 250 mM imidazole. Different portions of each fraction including 5 μl/2 μl of insoluble material (lane 1), 10 μl/36 ml of soluble material (lane 2), 10 μl/36 ml unbound material (lane 3), 15 μl/3 ml of wash 1 (lane 4), wash 2 (lane 5), and 15 μl/0.75 ml E1, E2, E3, and E4 (lanes 6–9), 10 μl/750 μl of 40% ammonium sulfate precipitate of pooled E1–E4 (lane 10) were analyzed on SDS–PAGE and stained with Coomassie (A) or subjected to Western blotting using avidin conjugated with alkaline phosphatase (B).

293T cells overexpressing PC i.e., 10 U/mg. The slight difference of PC activity could well be due to the purity of the enzyme used (50% pure in this study and 60% pure in our previous study) [8].

It is interesting to note that the endogenous Sf9 PC was not co-purified with the 6× His-hPC, therefore this system provides an excellent expression system to

produce mutant forms of human PC that are free from the endogenous Sf9 PC. Carbone et al. [9] have recently reported the expression of A610T hPC mutant in skin fibroblasts devoid of endogenous PC (PC^0) using a retroviral expression system. The expression in Sf9 cells reported here also provides an advantage over the mammalian expression system in which the non-6× His tagged recombinant human PC cannot be purified from endogenous PC [9]. As Sf9 cells are not serum-dependent as are 293T cells, this will allow a reduction in the cost of production of substantial amounts of recombinant human PC.

Acknowledgments

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