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Component co-expression and purification of recombinant human pyruvate dehydrogenase complex from baculovirus infected SF9 cells

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ABSTRACT

The mammalian pyruvate dehydrogenase complex (PDC) is a multi-component mitochondrial enzyme that plays a key role in the conversion of pyruvate to acetyl-CoA connecting glycolysis to the citric acid cycle. Recent studies indicate that targeting the regulation of PDC enzymatic activity might offer therapeutic opportunities by inhibiting cancer cell metabolism. To facilitate drug discovery in this area, a well defined PDC sample is needed. Here, we report a new method of producing functional, recombinant, high quality human PDC complex. All five components were co-expressed in the cytoplasm of baculovirus-infected SF9 cells by deletion of the mitochondrial localization signal sequences of all the components and E1a was FLAG-tagged to facilitate purification. The protein FLAG tagged E1a complex was purified using FLAG-M2 affinity resin, followed by Superdex 200 sizing chromatography. The E2 and E3BP components were then Lipoylated using an enzyme based *in vitro* process. The resulting PDC is over 90% pure and homogenous. This non-phosphorylated, lipoylated human PDC was demonstrated to produce a robust detection window when used to develop an enzyme coupled assay of PDHK.

Introduction

The pyruvate dehydrogenase complex (PDC)[1] catalyzes the irreversible oxidation of pyruvate to acetyl coenzyme A (acetyl-CoA), linking glycolysis to the tricarboxylic acid cycle (TCA) [1–3]. As befitting such a critical metabolic enzymatic machine, PDC is highly regulated primarily by reversible phosphorylation by PDH kinases (PDHKs) and phosphatases (PDPs) [4,5]. Aberrant PDC functioning either through normal aging or through the imposition of congenital or acquired diseases displays strikingly similar pathology to age-associated conditions such as sarcopenia, glucose intolerance, neurodegenerative diseases and cancer [6,7]. PDC and its regulatory kinases have thus been designated as potential therapeutic targets in multiple drug discovery efforts.

The PDC is composed of multiple copies of three distinct enzymes: the heterotetrameric (α2/β2)pyruvate dehydrogenase (E1), dihydrolipoamide acetyltransferase (E2), and dihydrolipoamide dehydrogenase (E3) [8–10]. In higher eukaryotes, PDCs have an additional structural component, E3-binding protein (E3BP) which tethers E3 within the PDC [11,12]. Human PDC contains multiple copies of E2 and E3BP that form a central core surrounded by non-covalently bound E1 heterotetramer and E3 [13,14]. E1 heterotetramer catalyzes the thaiminediphosphate dependent oxidative decarboxylation of pyruvate and the reductive acetylation of a lipoyl residue covalently attached to the lipoyl domain of E2 to generate a stable intermediate. E2 then catalyzes transfers of the acetyl group to CoA, leaving a reduced E2 lipoyl group that E3 uses as an electron source for the FAD-dependent reduction of NAD+ to NADH. Mitochondrial lipoyl-transferase catalyzes the lipoylation of E2, and this modification is essential for PDC activity [15,16].

In addition to being a therapeutic target, PDC is the physiological substrate of PDHKs and thus is often used as a substrate to screen for PDHK inhibitors in high throughput screening efforts in drug discovery programs. PDHK phosphorylation of PDC is directly correlated with PDC activity, so it can be measured by monitoring the reduction of NAD+ to NADH spectrophotometrically [17]. The assay requires homogenous, non-phosphorylated PDC as a substrate for high throughput screening and profiling campaigns. Currently available methods for the expression and purification of non-phosphorylated PDC can be cumbersome and suffer from a lack of reproducibility due to the nature of the host system (i.e. human tissues) [18–24]. Isolation of PDC complex from mammalian tissue results in low purity, sample heterogeneity, and some degree of phosphorylation [25–28]. Over-expression individually of each recombinant PDC component in *E. coli*

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[1] *Abbreviations used:* BSA, bovine serum albumin; HTS, high throughput screening; LPLA, lipoate protein ligase A; PDC, pyruvate dehydrogenase complex; PDHK, pyruvate dehydrogenase kinase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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followed by *in vitro* reconstitution of the PDC and further purification of the reconstituted complex is possible, but this results in relatively poor yields of the desired non-phosphorylated PDC, unable to support drug discovery scale assay development and screening platforms [29–34].

We report here a robust and high yielding strategy for the production of recombinant, non-phosphorylated, homogeneous PDC. The E1 heterotetramer, E2, E3, and BP subunits were co-expressed via baculovirus infection of insect SF9 cells. The E1α component of the E1 heterotetramer was engineered with an N-terminal FLAG affinity tag to enable an efficient 2-step purification protocol that yielded >90% pure, homogeneous, non-phosphorylated PDC. The co-expression in insect cells occurred in the cytoplasm, minimizing endogenous PDHK phosphorylation of PDC and sample heterogeneity, since PDHKs are predominantly localized to the mitochondria. The PDC complex was then lipoylated *in vitro* on the E2 subunit to ensure biological activity of the recombinant PDC complex. We have demonstrated a novel expression and purification method for the production of constitutively active, non-phosphorylated, homogeneous, recombinant human PDC to enable high throughput screening and drug discovery efforts on these important therapeutic targets. Furthermore, the successful expression and purification of an endogenous human mitochondrial protein complex utilizing insect cell expression reported here may be broadly applicable to other biologically and therapeutically interesting mitochondrial proteins.

Materials and methods

Materials

Pfu turbo DNA polymerase was obtained from Stratagene (La Jolla, CA). pFastBac1, pDONR221, pENTR-TEV/D-TOPO, pDEST 8 vectors, and BP/LR clonase were purchased from Invitrogen, Carlsbad CA. Ni–NTA agarose, DNA purification and agarose gel band extraction kits were obtained from Qiagen (Valencia, CA). Superdex 200 was purchased from GE Healthcare Biosciences (Piscataway, NJ). Dithiothreitol (Sigma Aldrich, D-5545), Adenosine-5′-triphosphate (ATP, Sigma Aldrich, A-6419), (±)-α-Lipoic acid (Sigma Aldrich 62320-5G-F) and anti-FLAG M2 Affinity gel (Sigma Aldrich, A2220). PDC extracted from porcine heart tissue (Sigma Aldrich P7032). Bio-Rad DC protein assay kit was used to quantity protein. All other laboratory chemicals and materials were of standard laboratory grade.

The Plasmids and expression

The five components of human PDC complex were subcloned into baculovirus expression vector with their N-term mitochondria localization signal sequence removed, respectively. The expression constructs for PDHE1a (30–390), PDHE1b (31–359), DLAT (87–647), PDHX (54–501), and DLD (36–509) (UniProtKB/Swiss-Prot Accession Nos. P08559, P11177, P10515, O00330, and P09622, respectively) were generated without tags. In addition, constructs for PDHE1a (30–390) and PDHE1b (31–359) were also generated with N-term FLAG His6 tag, respectively. The recombinant baculovirus was generated using the Bac-to-Bac baculovirus expression system (Invitrogen 10359-016). Both P1 virus and baculovirus-infected insect cells (BIICs) were generated. PDC complex expression was achieved by co-expression of all 5 components with either tagged PDHE1a (30–390) or tagged PDHE1b (31–359) in SF9 cells using BIICs [35]. Exponentially growing SF9 cells @ 2×10^6 cells/ml were infected with 10,000-fold diluted BIIC stock of each component. Equal amount of BIIC stock was used for all five components. Expression was done by co-expression of all 5 components with either tagged PDHE1a (30–390) or tagged PDHE1b (31–359). SF9 cells @ 2×10^6 cells/ml were infected with $\sim 1 \times 10^6$ BIIC (baculovirus-infected insect cells) of each component per 1 L of culture. The culture was incubated @ 27 °C, 90 rpm. Cell paste was harvested @ ~67 h post infection. Once the conditions were optimized, the process was scaled up to 20 L of culture.

E. coli lipoate-protein ligase A (LplA) (UniPort KB/SwissProt Accession No. P32099) was PCR amplified from the genomic DNA of DH5alpha strain [36]. The PCR product was subcloned into vector pENTR/TEV/D-TOPO (Invitrogen K252520). The entry clone was then transferred to a Gateway destination vector pDEST.T7.His6 (N-term tagged) by recombination. LplA was expressed in BL21/DE3 cells and induced with 0.5 mM IPTG for 20 h at 18 °C.

Recombinant PDHK1 (29–436) was expressed and purified as described previously [37].

Purification of recombinant FLAG-His E1a PDC complex

Cells paste of 20 L cell culture were lysed in lysis buffer (50 mM potassium phosphate, pH 7.4, 0.5 mM EDTA, 2 mM β-mercaptoethanol, 100 mM KCl) supplemented with complete protease inhibitors (Roche). The suspension was passed through an Avestin pressure drop homogenizer at 12 kpsi twice. The centrifugation at 30,000*g* for 45 min was performed to remove cell debris. The supernatant was collected for further use. The anti-FLAG resin was prewashed with 0.1 M glycine (pH 3.5) and equilibrated in buffer A (50 mM potassium phosphate, pH 7.4, 0.5 mM EDTA, 2 mM b-mercaptoethanol, 100 mM KCl). The equilibrated anti-FLAG resin was added to the supernatant to rotate for 2 h. After incubation, the supernatant was centrifuged at 3000*g* for 5 min to collect resin. The resin which captured target proteins was re-suspended and packed into XK26 column with buffer A. The column was washed by buffer A in about 5 CV until baseline to remove the contaminants. The PDC was eluted with 100 μg/ml FLAG peptides which is in buffer B (50 mM potassium phosphate, pH 7.4, 0.5 mM EDTA, 2 mM b-mercaptoethanol, 100 mM KCl). The fractions of elution peak were analyzed by SDS–PAGE. The fractions containing target proteins were pooled together as FLAG M2 elution pool. The pooled fractions were concentrated using 50-kDa molecular weight cut off centrifugal concentrator to a protein concentration of ~5–10 mg/mL. Preparative gel filtration was performed using a 320 mL Superdex 200 26/60 column. The column was equilibrated with buffer A in 1.5 CV. The concentrated PDC sample was injected in 10 ml and was eluted with buffer A. All fractions were detected and analysis by 4–12% gradient SDS–PAGE. The fractions containing PDC were pooled together based on the result of SDS–PAGE analysis. The purity of PDC complex was determined by gel densitometry. All purification steps were carried out at 4 °C, or with ice cold buffers. Chromatography was performed using ATKA purifier.

LCMS analysis

Mass spectrometry analysis was performed using a reversed-phase HPLC column (Agilent, Foster City, CA) coupled to a Q-TOF mass spectrometer. Mass reconstruction from raw data was accomplished with the software provided with the instrument.

Peptide mapping was carried out by the GSK Proteomics facility. Tryptic digests were analyzed on an Agilent MSD ion trap mass spectrometer. Protein identities were obtained from a search of the results using Mascot to search the PSR database.

Protein concentration determination

The concentration of purified PDC complex was determined by the Bradford method, using bovine serum albumin (BSA) as a standard [38].