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Towards the development of an enzyme replacement therapy for the metabolic disorder propionic acidemia

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ABSTRACT

Propionic acidemia (PA) is a life-threatening disease caused by the deficiency of a mitochondrial biotin-dependent enzyme known as propionyl coenzyme-A carboxylase (PCC). This enzyme is responsible for degrading the metabolic intermediate, propionyl coenzyme-A (PP-CoA), derived from multiple metabolic pathways. Currently, except for drastic surgical and dietary intervention that can only provide partial symptomatic relief, no other form of therapeutic option is available for this genetic disorder. Here, we examine a novel approach in protein delivery by specifically targeting and localizing our protein candidate of interest into the mitochondrial matrix of the cells. In order to test this concept of delivery, we have utilized cell penetrating peptides (CPPs) and mitochondria targeting sequences (MTS) to form specific fusion PCC protein, capable of translocating and localizing across cell membranes. In vitro delivery of our candidate fusion proteins, evaluated by confocal images and enzymatic activity assay, indicated effectiveness of this strategy. Therefore, it holds immense potential in creating a new paradigm in site-specific protein delivery and enzyme replacement therapeutic for PA.

1. Introduction

Metabolic disorders are life-threatening diseases caused by insufficient activities of enzymes required for the catabolism of metabolites that arise from the normal turnover of cellular constituents. One such disease is propionic acidemia (PA), which is an inherited autosomal recessive inborn error of metabolism during the neonatal period [11,12,43]. It affects approximately 1 in 30,000 live births worldwide [48] and PA patients may exhibit clinical symptoms such as protein intolerance, vomiting, lethargy, profound metabolic acidosis and mental retardation which can be sufficiently severe to cause death at young age [22]. These symptoms are caused by the deficiency of a mitochondrial biotin-dependent enzyme known as propionyl coenzyme-A carboxylase (PCC). PCC is responsible for the catabolism of propionyl coenzyme-A (PP-CoA), a metabolic intermediate arising from the normal turnover of several essential amino acids, as well as odd chain fatty acids in the matrix of mitochondria. The native PCC holoenzyme is composed of six α (PCCA) and six β (PCCB) subunits with the molecular weights of 72 kDa and 56 kDa respectively [25,46]. Deficiency in either or both PCC subunits and the consequent accumulation of PP-CoA leads to the pathogenesis of PA [36]. There is currently no cure for the deficiency of PCC and patients are managed by symptomatic treatment such as low-protein-high-energy diet or supplementation with specific mixtures free of propiogenic substrates, vitamins, and trace elements [8]. However, these treatments only partially improve symptoms and the overall outcome of severe forms of PA remains disappointing. Therefore, there is a need for developing an alternative durable and safe treatment for PA patients.

To date, more than 130 proteins and peptides are used as therapeutics in United States and European countries [31]. Enzyme replacement therapy (ERT) is a therapeutic approach which aims to restore the activity of a particular enzyme or protein in patients in cases of deficiency or abnormal production [29]. ERT has been studied in various metabolic enzyme deficiencies such as Gaucher's, Hurler's, Fabry's, Pompe's disease, and in Maroteaux-Lamy syndrome to reverse the pathogenesis of the chief clinical manifestations of these diseases [1,45,16,6,20,28,52]. With the large number of studies on ERT, one can anticipate that ERT will have an expanding role in the treatment of rare metabolic diseases. In this paper, we examine the possibility of developing an enzyme replacement strategy for the relief of PA.

Physicochemical factors such as delivery and stability of protein are classical challenges to the development of ERT for PA patients [31,34]. One of the key challenges to effective ERT is the plasma membrane of living cells, which is a formidable barrier to permeability by exogenous molecules. In the case of developing protein therapeutics for the

Abbreviations: PA, propionic acidemia; PCC, propionyl coenzyme-A carboxylase; PP-CoA, propionyl coenzyme-A; CPPs, cell penetrating peptides; MTS, mitochondria targeting sequences; ERT, enzyme replacement therapy; MPP, mitochondrial processing peptidase; LAD, lipoamine dehydrogenase; PCCA, PCCα subunit; PCCB, PCCβ subunit; His-tag, six histidines tag; UPLC-MS/MS, ultra performance liquid chromatography tandem mass spectrometry; CoA, coenzyme-A.

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treatment of PA, the delivery of an effective therapeutic protein requires the delivery of the protein across the cell membrane, followed by a subsequent targeting of the protein into the mitochondria of the cells poses an additional level of challenge. To overcome the challenge of delivering and targeting proteins into the mitochondria of living cells and stabilize them in ERT, two groups of peptides called cell penetrating peptides (CPPs) and mitochondrial targeting sequences (MTS) were examined. Recent studies used CPP as an approach to overcome the cell's plasma membrane barrier to deliver recombinant enzymes into the cells and their organelles such as mitochondria [9,24,26,47]. The most well-studied CPP is a peptide derived from human immunodeficiency virus transactivator of transcription (HIV-1 TAT), known as the TAT peptide. TAT is an 11-amino acid domain and has been reported to translocate through cell membranes in a receptor-independent fashion either alone or linked to bulky peptides and proteins cargoes [14,17,33]. On the other hand, MTS are typically amino-terminal (N-terminal) amphipathic α-helices targeting signal peptides which are around 15–50 amino acid residues in length. They direct mitochondrial proteins to the mitochondrial matrix upon translation and are removed by a mitochondrial processing peptidase (MPP) upon import [4,18,19].

Both TAT and MTS have been investigated for their applicability in delivering biologically active fusion proteins to cells and organelles in various murine tissue [3,15,50,55] (for recent reviews see [5,21,54]). More recently, Rapoport et al. used protein transduction strategy to develop an approach for restoring the activity of human lipoamine dehydrogenase (LAD) enzymatic complex in a mitochondrial disorder known as LAD deficiency through the utilization of a TAT-MTS-LAD fusion protein [41]. Their result suggested that expressed and purified fusion protein could be successfully delivered into the mitochondria of deficient cell lines and tissue of deficient mice to restore the activity of an essential mitochondrial enzymatic complex [41,42]. Moreover, numerous groups have successfully exploited the ability of both TAT and MTS fusion domains for transduction of the recombinant mitochondrial proteins across mitochondrial membranes in in vitro and in vivo studies [9,10,23,47]. These pieces of evidences suggested that TAT fusion proteins could cross both cellular and mitochondrial membranes, and that incorporation of a MTS into a TAT fusion protein would allow processing and localization of the exogenous proteins in mitochondria. It is thus suggested that TAT and MTS fusion proteins may represent a viable option as potential mitochondrial protein therapies for direct delivery of ERT into patients. However, targeting of any new protein into mitochondria using the strategy described above requires detailed examination and optimization.

To our knowledge, there has not been any report of functionally active proteins that are specifically targeted or delivered to the mitochondrial matrix as a therapeutic approach for PA. In this paper, we propose the combined utilization of TAT and MTS for transduction of mitochondrial enzyme subunits, PCCA and PCCB, into mitochondria of cells as a first step to the development of a site-specific ERT for PA.

2. Materials and methods

Oligonucleotide primers were ordered from 1st Base (Singapore, Singapore). GelRed® nucleic acid gel stain, MitoView 633 fluorescent mitochondrial dye, and Mix-n-Stain™ CF488A were purchased from Biotium (Hayward, CA). Restriction endonucleases (*Hin*dIII-HF, *Not*I-HF, *Eco*RI-HF and *Bam*HI-HF) and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA), while KOD DNA polymerase was purchased from Novagen-EMD4 Biosciences (Darmstadt, Germany). Mitochondrial isolation from mammalian cells and enzymatic activity assay were performed as described previously [7].

2.1. Construction of plasmids for expression of fusion protein variants

cDNA of PCC α (pccA) and β (pccB) subunit were ordered to Geneart (San Francisco, CA) to be constructed based on the mature protein sequences reported by Kelson et al. [30]. In order to construct the recombinant pccA and pccB genes fused to MTS-TAT, TAT or MTS domains on their C-terminal site, full length cloned pccA and pccB genes were used as templates in PCR assay. Fusion domains were constructed to be flanked by glycine spacer residues. The details of oligonucleotides used as forward and reverse primers are listed in Supplementary material Tables 1 and 2. All reverse primers were designed to have complementary segments with melting temperature (Tm) at around 68 °C to allow stepwise amplification. Primers were designed using Jellyfish software version 3.3.1 (Field Scientific, Lewisburg, PA). PCR reaction mixture (50 μl) contained 10 nM of each of primers and 0.5 μl of KOD DNA polymerase. The PCR products for pccA and pccB fusion constructs were digested by *Eco*RI-HF/*Hin*dIII-HF and *Bam*HI-HF/*Not*I-HF endonucleases respectively before ligation into pET28a fusion expression vector (Merck Millipore Division, Merck Pte. Ltd., Darmstadt, Germany), which adds six histidines tag (His-tag) on N-terminal of proteins. pccA and pccB plasmid constructs were transformed into *E. coli* DH5α competent cells. N-terminal fusion subunits have been previously prepared from pccA and B dodecamer variants, which were constructed by Geneart (NC, USA). All constructs were sequenced by 1st BASE (Singapore, Singapore) for the presence of the intact TAT, MTS and MTS-TAT on their corresponding C or N terminals.

2.2. Recombinant expression and purification of human PCC subunits

E. coli BL21 (DE3) competent cells were transformed with plasmids encoding the N-terminal or C-terminal fusion PCC subunits. The transformed cells were grown at 37 °C in 1 L LB medium containing 50 μg/ml kanamycin with addition of 5 μM biotin for PCCA variants expression. Protein expression was induced by adding isopropyl-β-D thio galactopyranoside (IPTG) to culture with $OD_{600\ nm}$ of 0.6–0.8 to a final concentration of 1 mM. Induced cells were allowed to grow overnight at 18 °C before collection. The cells were harvested by centrifugation (7000 rpm for 20 min at 4 °C) and the pellets were sonicated in pulses using denaturing lysis buffer (50 mM NaH_2PO_4, 30 mM NaCl, 10 mM imidazole, 8 M urea, pH adjusted to 8) for 20 min. The suspensions were clarified by centrifugation (13,000 rpm for 30 min at 4 °C), and the supernatant containing the denatured fusion proteins were loaded on a 5 ml His-Trap nickel columns from GE Healthcare (Uppsala, Sweden). The columns were washed by stepwise addition of increasing imidazole from 10 mM to 500 mM and finally eluted with elution buffer (50 mM NaH_2PO_4, 30 mM NaCl, 500 mM imidazole, 8 M urea, pH adjusted to 8). The purification procedures were carried out using the ÄKTA Prime Plus system (GE Healthcare, Uppsala, Sweden). Urea and other salts were removed by Hi-prep 26/10 desalting column (GE Healthcare, Uppsala, Sweden). Aliquots of the proteins at 80% purity desalted against phosphate buffered saline (PBS) or TAE buffer (40 mM Tris–HCl, pH 8.0, 0.1 mM EDTA) were snapped frozen in liquid nitrogen and were kept in −80 °C until use.

2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis

Protein concentrations were calculated by Bradford dye assays. Around 10–30 μg protein per lane were resolved on 12% polyacrylamide SDS-PAGE. Gels were subsequently stained with InstantBlue™ (Expedeon Ltd., Cambridge, UK) to visualize the protein bands. Western blot analysis was performed using anti-PCCB mouse polyclonal (I-DNA Biotechnology Pte-Ltd., Singapore, Singapore), anti-PCCA chicken polyclonal (Sigma-Aldrich, St. Louis, MO) and anti-E1α (Invitrogen, Carlsbad, CA) as primary antibodies and anti-mouse HRP conjugated IgG (goat), and anti-chicken HRP conjugated IgG (bovine) (Santa Cruz Biotechnology, Santa Cruz, CA) as secondary antibodies at 1:1000 dilutions. Densitometric analysis of western blot's bands of target PCC subunits was conducted using National Institutes of Health (NIH) ImageJ 1.47 software.