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Recombinant expression and characterization of biologically active protein delta homolog 1

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ABSTRACT

Obesity is characterized by an excessive accumulation of body fat, for which impaired adipogenesis is believed to play a crucial role. As a gatekeeper of early adipogenesis, protein delta homolog 1 (DLK1) has a pivotal role in deciding whether pre-adipocytes will differentiate, determining the balance between healthy and unhealthy fat tissue. Here, an expression system for the cysteine-rich soluble human DLK1 was established. DLK1 was overexpressed in *Escherichia coli* BL21(*DE3*)pLysRARE, purified by affinity chromatography and refolded by stepwise dialysis. Identity, purity, secondary structure and refolding efficiency were determined. Proteolytic digestion followed by mass spectrometry analysis proved correct disulfide bridge formation. The biological activity of DLK1 was examined by differentiation assays in murine pre-adipocyte-like 3T3-L1 cells. Thereby, recombinantly produced DLK1 was shown to inhibit adipogenesis in a concentration- and time-dependent manner. All in all, our approach gives access to large amounts of active DLK1 and can be transferred to related proteins.

Introduction

Obesity is defined as an excessive and abnormal accumulation of body fat. Beside direct negative effects, like reduced mobility, social stigmatization, depression and other mental disorders, obesity is a major risk factor for comorbidities such as cardiovascular diseases, type 2 diabetes and different types of cancer [1].

Contrary to previous assumptions, the function of adipocytes is not limited to fat storage, but they also act as important secretory cells for a variety of different hormones, known as adipokines. In obese subjects, either adipocyte hyperplasia (increase in number) or hypertrophy (increase in size) lead to an increase in the size of the adipose tissue. Currently it is suggested that an increase in adipocyte size leads to an exceeded lipid storage capacity of the fat tissue and a lipid spill over to other tissues such as liver or pancreas, which might subsequently cause fat liver disease or diabetes, respectively. In contrast, hyperplasia is associated with a metabolically healthy obese phenotype [2]. Hence, a proper regulation of the amount of adipocytes (and thereby regulation of their size, or *vice versa*) is a critical point in maintenance of a healthy metabolism even in situations of nutritional overload. Adipogenesis, the differentiation of precursor cells called pre-adipocytes into mature adipocytes, is a major process to adjust adipocyte number. It is tightly regulated by activation of protein cascades, including nuclear receptor proteins from the peroxisome proliferator-activated receptor (PPAR)[1] family and different CCAAT-enhancer-binding proteins (C/EBP) [3]. As one of the first steps in these cascades, downregulation of protein delta homolog 1 (DLK1) takes place.

Human DLK1 consists of 383 amino acids. It is expressed as a membrane-bound form and released after protease cleavage. The resulting soluble DLK1 (amino acids 24-303, also known as fetal antigen 1 (FA1)) acts as para- and autocrine signal. It consists of six EGF-like repeats, each containing six cysteines that form three conserved disulfide bridges [4]. DLK1 is widely expressed in the fetal organism but only present in few adult tissues, including ovaries, placenta, pituitary and adrenal tissues [5]. A loss of DLK1 expression, as shown in mice knock-out experiments, results in an accelerated fat deposition, a dramatic increase in serum lipid metabolites and an obese phenotype [6]. In contrast, mice overexpressing DLK1 in the adipose tissue showed a decrease in fat mass and a reduced expression of adipocyte markers and adipocyte-secreted factors, but suffer from hypertriglyceridemia, decreased

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[1] *Abbreviations used:* DLK1, protein delta homolog 1; PPAR, peroxisome proliferator-activated receptor; C/EBP, CCAAT-enhancer-binding protein; FA1, fetal antigen 1; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; BSA, bovine serum albumin; Ni-NTA, Ni^{2+}-nitrilotriacetate; TFA, trifluoroacetic acid; CD, circular dichroism; FCS, fetal calf serum; PC, positive control; NC, negative control; ANOVA, analysis of variance; DLL1, Delta-like 1.

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insulin sensitivity and reduced glucose tolerance [7]. Besides its effects in adipogenesis, DLK1 also influences remodeling of the tumor blood vasculature [8], modulates tumorigenesis [9–12] and regulates myogenesis in skeletal muscle [13] as well as in C2C12 cells [14].

Despite its clinical relevance, the mode of action of DLK1 is not fully understood. Therefore, an expression system for the production of large amounts of recombinant DLK1 can be a basis for better understanding its role in diseases like obesity and cancer. Hence, the aim of this work was to express the soluble form of DLK1 as His-tagged fusion protein in *Escherichia coli* BL21(*DE3*)pLysRARE, and, subsequently, to purify and refold it in order to obtain sufficient amounts of active protein. After characterization with HPLC, MALDI-MS and circular dichroism spectroscopy, DLK1 was tested in adipocyte differentiation assays on 3T3-L1 cells. A concentration-dependent effect of recombinantly produced DLK1 could be demonstrated. Moreover, the effect of DLK1 was highest when given at an early stage of differentiation, whereas a treatment in the later differentiation process had no effect.

Material and methods

Plasmid construction

DLK1 cDNA clone was purchased from imaGenes (Clone IRAUp969D0133D). The coding sequence of soluble DLK1 without its signal sequence (UniProt entry P80370, amino acids 24-303 [15]) was amplified by PCR using the forward primer 5′-GCCCGGCATATGGCTGAATGCTTCCCGG-3′ adding a restriction site for NdeI and the reverse primer 5′-GCACGGGGATCCTCACTGGCC CTCGGTGAGG-3′ adding a restriction site for BamHI (underlined sequences showing the recognition sites for the restriction endonucleases). The obtained construct was cloned into the pET-16b expression vector (Merck) using NdeI and BamHI as restriction endonucleases. The final vector pET-16b_DLK1 allowed expression of His_{10}-tagged fusion protein.

Bacterial strains and transformation

E. coli DH5α was used as bacterial host for plasmid construction and amplification and *E. coli* BL21(*DE3*)pLysRARE was used for bacterial expression of recombinant protein.

Transformation of chemically competent bacteria (RbCl method [16]) was done by a 90 s heat shock (42 °C) followed by 60 min incubation at 37 °C. The transformed bacteria were plated on ampicillin-containing agar plates. Single colonies were picked and incubated overnight in LB medium. 850 µl bacteria solution and 150 µl glycerol were mixed and stored as glycerol stock at −70 °C.

Protein expression

50 ml TB/ampicillin (100 µg/ml) were inoculated with the glycerol stock of transformed *E. coli* BL21(*DE3*)pLysRARE as a starter culture and were grown for 16 h at 37 °C.

1 L TB/ampicillin (100 µg/ml) was inoculated with the starter culture to an OD_{600} of 0.1. The culture was further incubated at 37 °C until the OD_{600} reached 0.6 when the expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG, 1 mM final concentration). DLK1 was expressed for 6 h and cells were harvested by centrifugation, resulting in 4.3 g of wet weight cells. Cells were resuspended in 40 ml lysis buffer (20 mM HEPES, 500 mM NaCl, 5 mM $MgCl_2$, DNase I, pH 8) and lysed by French Press (SLM Aminco). Inclusion bodies were pelleted and washed with HEPES buffer (20 mM HEPES, 500 mM NaCl, pH 8), two times with 3 M urea buffer (3 M urea, 20 mM HEPES, 500 mM NaCl, 0.5% Triton X-100, pH 8) and again HEPES buffer. Subsequently inclusion bodies were solubilized with 8 M urea buffer (8 M urea, 20 mM HEPES, 500 mM NaCl, 25 mM 2-mercaptoethanol, pH 8) and cell debris was removed by centrifugation at 18,000*g* for 30 min at 4 °C.

Protein purification and refolding

The solubilized protein was diluted with HEPES buffer to 6 M urea and loaded on Ni-NTA (Ni^{2+}-nitrilotriacetate)–agarose-packed columns (Qiagen). After washing with HEPES buffer, His_{10}-tagged DLK1 was eluted by 6 M urea buffer (6 M urea, 20 mM HEPES, 500 mM NaCl, pH 8) supplemented with stepwise increasing concentrations of imidazole (10–500 mM). Elution fractions containing protein were pooled, diluted to 4 M urea and 0.1 mg/ml protein, and 100 mM 2-mercaptoethanol were added. 20 ml of this solution was refolded by stepwise dialysis against a 100-fold excess of refolding buffer I (4 M urea, 20 mM HEPES, 500 mM NaCl, pH 8), II (3.5 M urea, 20 mM HEPES, 500 mM NaCl, pH 8), III (2.5 M urea, 20 mM HEPES, 500 mM NaCl, 0.5 M L-arginine, 1 mM EDTA, pH 8), IV (1.25 M urea, 20 mM HEPES, 500 mM NaCl, 0.5 M L-arginine, 1 mM EDTA, 10 mM cysteine, 1 mM cystine, pH 8), V (0.5 M urea, 20 mM HEPES, 500 mM NaCl, 0.25 M L-arginine, 1 mM EDTA, 10 mM cysteine, 1 mM cystine, pH 8), VI (20 mM HEPES, 500 mM NaCl, 1 mM EDTA, 10 mM cysteine, 1 mM cystine, pH 8), VII (20 mM HEPES, 500 mM NaCl, pH 8) and VIII (20 mM HEPES, 500 mM NaCl, pH 8), respectively. Every step was stirred for 2 days at 4 °C. After the final dialysis step, refolded protein was concentrated by ultra centrifugal filters (Amicon) to a concentration of approximately 5 mg/ml and stored at −20 °C.

For differentiation assays of non-folded protein, DLK1 purified by IMAC was incubated with 500 equivalents iodoacetamide 30 min at room temperature in the dark. Hereafter, 50 mM 2-mercaptoethanol were added and this solution was dialyzed two times against a 500-fold excess of HEPES buffer for 1 day at 4 °C.

Protein analysis

Protein concentrations were determined by Bio-Rad Protein Assay (BIO-RAD) in triplicate against a bovine serum albumin (BSA) standard. Concentration of pure protein was calculated using the Lambert–Beer law. Measurement of absorbance at 280 nm was performed with a NanoQuant Plate (d = 0.05 cm) and an Infinite 200 microplate reader (Tecan). The molar extinction coefficient ε at 280 nm (20,700 M^{-1} cm^{-1} for refolded DLK1, 18,450 M^{-1} cm^{-1} for non-folded DLK1) was calculated using ProtParam [17].

For SDS–PAGE, samples were run on a 12% polyacrylamide gel and stained with naphthol blue black. For western blots, proteins from a SDS gel were transferred to an Amersham Hybond-P PVDF membrane (GE Healthcare) by semi-dry blotting. The membrane was blocked for 1 h with 5% BSA in TBS–Tween buffer (TBS buffer, 0.5% Tween 20) and incubated with primary antibody (HRP-coupled-Anti-6X His tag® antibody (abcam) or anti-DLK1 MaxPab mouse polyclonal antibody (Abnova), 1:500 in TBS–Tween, 0.1% BSA) overnight. After washing three times with TBS–Tween buffer, membrane was incubated with secondary antibody (HRP-coupled goat anti-mouse antibody (Santa Cruz), 1:5000 in TBS–Tween, 0.1% BSA) for 1.5 h, washed with TBS–Tween buffer three times and developed with ECL Western Blotting Substrate (Pierce) and G:BOX ChemieXL camera (Syngene).

Samples for HPLC were diluted 1:4 in solvent A (H_2O, 0.1% trifluoroacetic acid (TFA)) and separated on a reversed-phase C18 column (Grace Vydac RP18 column, 4.6 mm × 250 mm, 5 µm particle size, 300 Å pore size) with a gradient of 20–100% of solvent B (acetonitrile, 0.08% TFA) in solvent A in 60 min.

Mass spectra of purified recombinant proteins were obtained in linear mode on a MALDI-TOF/TOF UltraflexIII (Bruker Daltronics).