Archives of Biochemistry and Biophysics 618 (2017) 45–51

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

A scalable lysyl hydroxylase 2 expression system and luciferase-based enzymatic activity assay

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ARTICLE INFO

Article history:
Received 9 December 2016
Received in revised form
6 February 2017
Accepted 13 February 2017
Available online 20 February 2017

Keywords:
Lysyl hydroxylase 2
Collagen
High-throughput assay
Succinate detection
Chinese hamster ovary cell
Oxygenase

ABSTRACT

Hydroxylysine aldehyde-derived collagen cross-links (HLCCs) accumulate in fibrotic tissues and certain types of cancer and are thought to drive the progression of these diseases. HLCC formation is initiated by lysyl hydroxylase 2 (LH2), an Fe(II) and α-ketoglutarate (αKG)-dependent oxygenase that hydroxylates telopeptidyl lysine residues on collagen. Development of LH2 antagonists for the treatment of these diseases will require a reliable source of recombinant LH2 protein and a non-radioactive LH2 enzymatic activity assay that is amenable to high throughput screens of small molecule libraries. However, LH2 protein generated using *E coli*– or insect-based expression systems is either insoluble or enzymatically unstable, and the LH2 enzymatic activity assays that are currently available measure radioactive CO_2 released from ^{14}C-labeled αKG during its conversion to succinate. To address these deficiencies, we have developed a scalable process to purify human LH2 protein from Chinese hamster ovary cell-derived conditioned media samples and a luciferase-based assay that quantifies LH2-dependent conversion of αKG to succinate. These methodologies may be applicable to other Fe(II) and αKG-dependent oxygenase systems.

1. Introduction

The hydroxylation of lysine residues on collagen catalyzed by lysyl hydroxylases (LHs; EC 1.14.11.4) is critical to the formation of covalent cross-links that connect collagen molecules and stabilize the extracellular matrix [1]. LHs belong to a superfamily of more than 60 oxygenases that use Fe(II) as a cofactor and α-ketoglutarate (αKG) and oxygen as co-substrates; these oxygenases include, among others, the collagen prolyl hydroxylases, the jumonji family of histone demethylases, hypoxia-inducible factor (HIF) prolyl hydroxylase domain-2, and asparaginyl hydroxylase factor inhibiting HIF-1α [2]. Three LH family members—LH1, 2, and 3—are found in vertebrate genomes, whereas only one LH family member that is homologous to vertebrate LH3 has been identified in invertebrates such as *Trichoplusia ni* and *Spodoptera frugiperda* [3]. LH2 has 3 potential isoforms, two of which (LH2a and LH2b) have been shown to be functional enzymes [4]. LH2b is the major isoform and is 21 amino acids longer than LH2a owing to the inclusion of 63 nucleotides in exon 13A that are alternatively spliced [4]. LH2b, hereafter abbreviated to LH2, is unique because it is the only LH family member known to modify telopeptidyl lysine residues [5–7]. The hydroxylated telopeptidyl lysines that result from LH2 modification are converted by lysyl oxidases into hydroxylysine aldehydes, which subsequently condense with juxtaposed lysines or hydroxylysines to form hydroxylysine aldehyde-derived collagen cross-

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http://dx.doi.org/10.1016/j.abb.2017.02.003
0003-9861/

links (HLCCs) [8]. HLCCs are resistant to collagenase cleavage, are more stable than lysine-derived collagen cross-links (LCCs) that form in the absence of LH2, and are particularly abundant in skeletal tissues such as cartilage and bone, which require high tensile strength. Genetic evidence supports a central role for LH2-mediated HLCC formation in normal bone function. Indeed, inactivating mutations in the procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 gene, *PLOD2*, which encodes LH2, are found in patients with Bruck syndrome [9]; these individuals have deformed, fragile bones and are deficient in HLCCs.

In fibrotic diseases, the extracellular matrices contain high levels of HLCCs and are aberrantly stiff [6,10]. Collagen rich in LCCs can be removed by collagenases; however, the immune response to tissue injury often increases LH2 expression [11], which leads to the sustained formation of HLCCs that are resistant to removal, causing scarring or fibrosis under pathological conditions. LH2 levels are increased in multiple types of fibrosis [6,10]. Moreover, in progressive fibrotic diseases such as idiopathic pulmonary fibrosis, the accumulation of collagen increases tissue stiffness and stimulates myofibroblast differentiation and additional collagen production, thereby activating a vicious cycle [12–16]. Thus, pharmacologic inhibitors of LH2 may stop or even reverse fibrotic disease progression.

We recently established a link between fibrosis and cancer progression and demonstrated a role of LH2 in lung cancer metastasis [17]. We showed that in patients with lung adenocarcinomas, intra-tumoral LH2 levels are prominently upregulated and predict a significantly shorter survival duration [17]. In addition, LH2 knockdown reduces HLCC-to-LCC ratios without changing the total amount of collagen cross-links, decreases tumor stiffness, and abrogates tumor cell migration, invasion, and metastasis [17]. We also found that LH2 is secreted by lung cancer cells and can modify collagen in the extracellular space [18]. LH2 promotes metastasis in other cancer types, including sarcoma [19] and breast cancer [20,21]. Thus, LH2 is a potential therapeutic target in cancer.

Therapeutically targeting LH2 will require a reliable LH2 expression system that enables biochemical and crystallographic analysis, as well as a robust high throughput assay for large-scale LH2 antagonist screening. Previous studies have demonstrated that LH family members cannot be expressed as a soluble protein in *E coli*, and insect cell–based protein expression systems, such as *Trichoplusia ni* high-five cells and *Spodoptera frugiperda* Sf9 cells, produce LH2 protein with unstable enzymatic activity [22,23], which may have resulted from the expression systems and/or purification procedures used [7]. However, several earlier studies successfully purified enzymatically active LH family members from chick embryos [24,25], which suggests that a vertebrate host is important for the expression of vertebrate LHs.

To quantify LH2 enzymatic activity, investigators have implemented a method that detects radioactive CO_2 released from ^{14}C-labeled αKG [26,27]. Although it produces a detectable signal from small amounts of LH enzyme, this assay is semi-quantitative and therefore is not optimal for high throughput screening. Non-radioactive methods that assess the enzymatic activity of Fe(II) and αKG-dependent oxygenases by measuring αKG diminution or succinate production have been developed [28,29]. Compared to assays that measure αKG diminution, succinate detection-based assays have shown superior sensitivity; however, the currently available assays still require large amounts of recombinant protein to generate a reliable signal in a hydroxylation reaction, which makes them impractical for carrying out large-scale inhibitor screening.

To enable the characterization and therapeutic targeting of LH2, here we developed a scalable method of purifying LH2 protein from Chinese hamster ovary (CHO) cell–conditioned media, developed a luciferase assay that measures LH2 activity on the basis of succinate production, and confirmed that the purified recombinant LH2 has stable enzymatic activity and that our assay has high signal-to-noise and minimal batch-to-batch variation.

2. Materials and methods

2.1. LH2 production and purification

LH2 was purified as described previously with minor modifications [30]. Briefly, human LH2 (residues 33–758, wild-type and inactive D689A mutant) recombinant proteins were produced from new Gibco™ ExpiCHO™ cells in suspension (Thermo Fisher Scientific, Waltham, MA) as a secreted protein with N-terminal His8 and human growth hormone (hGH) tags via large-scale transient transfection with polyethylenimine. The cells were transfected at a density of 2×10^6 cells/mL with 1 mg of DNA and 3 mg of polyethylenimine per liter of cells [30]. After 5 h, cells were split at a ratio of 2:9 and grown for 4 days. The LH2-containing conditioned media were then harvested by centrifugation at 7000 rpm for 10 min, filtered through 0.22 μm EMD Millipore Stericup™ Sterile Vacuum Filter Units (EMD Millipore, Billerica, MA), concentrated to 100 mL, and buffer-exchanged into Nickel-binding buffer (20 mM Tris, 200 mM NaCl, 15 mM imidazole, pH 8.0) using the Centramate™ & Centramate PE Lab Tangential Flow System (Pall Life Sciences, Ann Arbor, MI) at a flow rate of 100 ml per minute with a pressure of 20–30 psi. Using this system, a 15-L batch of conditioned medium is typically processed within 24 h. The recombinant LH2 proteins were then purified from CHO cell–conditioned media with immobilized metal affinity chromatography and anion exchange chromatography consecutively using NGC™ Medium-Pressure Liquid Chromatography Systems (Bio-Rad, Hercules, CA). CHO cell–conditioned media containing LH2 proteins were loaded into a Nickel column at a flow rate of 3 ml per min, washed with 10 bed volumes (50 mL) of Nickel-binding buffer followed by 6 bed volumes (30 mL) of nickel-binding buffer with 10 mM extra imidazole and then eluted with 400 mM imidazole in 200 mM NaCl, pH 8.0. The eluted LH2 proteins were diluted in water at a ratio of 1:4 and loaded into an anion exchange column at a flow rate of 3 ml per min. After the column was washed with 10 bed volumes of washing buffer (50 mM NaCl, 50 mM HEPES, pH 7.4), LH2 proteins were eluted with a linear gradient of up to 1 M NaCl, 50 mM HEPES, pH 7.4. The LH2 protein–containing fractions were collected, pooled, concentrated to 1 mg/mL with the Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-30 membrane (EMD Millipore, Billerica, MA), buffer-exchanged into reaction buffer (150 mM NaCl, 50 mM HEPES, pH 7.4), snap-frozen in liquid nitrogen, and stored at −80 °C. The protein concentration was determined with Nanodrop (Thermo Fisher Scientific) by measuring absorbance at 280 nm. The typical LH2 yield from a single 15-L batch of CHO cell culture was approximately 2 mg/L.

2.2. Protein gel electrophoresis and western blotting

Protein purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). LH2 proteins were visualized by staining the gels with SimplyBlue™ Safe Stain (Thermo Fisher Scientific). To confirm that the protein was LH2, we transferred the protein in the SDS-PAGE gel to polyvinylidene fluoride (PVDF) membrane using the *Trans*-Blot Turbo Transfer System and a *Trans*-Blot Turbo Mini PVDF Transfer Pack (Bio-Rad). The membrane was blocked with 5% milk in phosphate-buffered saline–Tween 20 (PBS-T; 0.01 M PBS, 0.1% Tween 20, pH 7.4) for 1 h at room temperature and incubated with antibodies against His6, hGH (sc-8036 and sc-10365, respectively, Santa Cruz, Dallas,