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Full-length myocilin protein is purified from mammalian cells as a dimer

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ABSTRACT

Myocilin (MYOC) is a secreted protein found in human aqueous humor (AH) and mutations in the *MYOC* gene are the most common mutation observed in glaucoma patients. Human AH analyzed under non-reducing conditions suggests that MYOC is not normally found in a monomeric form, but rather is predominantly dimeric. Although MYOC was first reported almost 20 years ago, a technical challenge still faced by researchers is an inability to isolate full-length MYOC protein for experimental purposes. Herein we describe two methods by which to isolate sufficient quantities of human full-length MYOC protein from mammalian cells. One method involved identification of a cell line (HeLa S3) that would secrete full-length protein (15 mg/L) while the second method involved a purification approach from 293 cells requiring identification and modification of an internal MYOC cleavage site (Glu214/Leu215). MYOC protein yield from 293 cells was improved by mutation of two MYOC N-terminal cysteines (C47 and C61) to serines. Analytical size exclusion chromatography of our full-length MYOC protein purified from 293 cells indicated that it is predominantly dimeric and we propose a structure for the MYOC dimer. We hope that by providing methods to obtain MYOC protein, researchers will be able to utilize the protein to obtain new insights into MYOC biology. The ultimate goal of MYOC research is to better understand this target so we can help the patient that carries a *MYOC* mutation retain vision and maintain quality of life.

1. Introduction

Corticosteroids (glucocorticoids) are utilized as potent anti-inflammatory agents. One troublesome potential side effect is elevated intraocular pressure (IOP) and increased risk of glaucoma [1]. The retinal ganglion cells and its axons that form the optic nerve are sensitive to IOP elevation, so glaucoma is characterized by cupping of the optic-nerve head and vision loss. To better understand steroid-induced IOP elevation, research in the late 1990s by two independent groups identified a gene highly-upregulated in response to steroid treatment [2,3] and they named the gene trabecular induced glucocorticoid response (*TIGR*). As the N-terminal of the TIGR protein shares approximately 25% identity with myosin, the gene was later renamed to myocilin (*MYOC*). Studies of MYOC promoter did not reveal a proximal glucocorcorticoid response element (GRE) in the MYOC promoter suggestive that steroids regulate MYOC expression indirectly and not by direct transcriptional regulation [4].

MYOC protein is comprised of 504 amino acids. At the N-terminal there is a secretion signal with a cleavage site at amino acid 32 [5] as well as a single *N*-glycosylation site at amino acid 57 [6]. By western blot only a portion of human MYOC protein appears to be *N*-glycosylated [6]. *N*-glycosylation means MYOC is being synthesized within the endoplasmic reticulum which is a cellular organelle containing numerous chaperone proteins which facilitate proper protein folding. At its N-terminal, MYOC possess a coil-coil region which contains a leucine zipper. A flexible-linker section connects the N-terminal region to the C-terminal five-bladed beta-propeller [7]. At the extreme C-terminus there is a –SKM sequence which has been proposed to be a cryptic peroxisome targeting signal [8]; however, with the C-terminal structure of MYOC now solved, we know that the –SKM sequence is not cryptic,

Abbreviations: AH, Aqueous humor; B-ME, Beta-mercaptoethanol; Ext-OLF, Extended Olfactomedin domain; FL, Full-length; IOP, Intraocular pressure; MW, molecular weight; MYOC, Myocilin; OLF, Olfactomedin; wt, Wild-type

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but rather it is fully exposed [7]. The C-terminal of MYOC has 40% identity to the nearest olfactomedin (OLF) domain family member. MYOC has been reported to be highest expressed in the eye, skeletal muscle, and heart [5,9,10].

MYOC is the gene with mutations most strongly-associated with glaucoma. *MYOC* mutations are reported in 2–4% of all glaucoma patients [11,12]. By 2020 it is estimated that the global glaucoma population will be approximately 80 million people [13] and that means the glaucoma patient population with a *MYOC* mutation will be greater than 3 million. To date, more than 70 pathologic *MYOC* mutations have been reported (myocilin.com). In the majority of cases, the pathologic mutation is in the C-terminal OLF domain. The most common MYOC mutation introduces an early stop codon, Q368X, and it is found in over one-third of glaucoma patients carrying a *MYOC* mutation [14]. One unique *in vitro* feature of pathologic MYOC C-terminal mutant proteins is that mutant protein is not secreted, but rather is retained within the cell [15,16].

Glaucoma patients with *MYOC* mutations are younger than the general glaucoma population [17] and have extremely-high IOPs [18]. This high IOP many not be lowered sufficiently by current IOP-lowering medications thus, requiring these patients to have IOP-lowering surgery. IOP-lowering surgeries are prone to failure [19], so these young patients will require numerous such surgeries during their lifetime. Despite intensive research efforts, the function of wild-type (wt) MYOC remains unknown and how mutant MYOC contributes to glaucoma disease pathology is ambiguous [20,21].

Part of the reason there is so much unknown regarding MYOC is that there are many challenges associated with studying MYOC *in vitro*. For example, MYOC protein is a secreted protein that is detected in primary trabecular meshwork cells, but its expression decreases with culture time [22] and most immortalized cell lines no longer produce endogenous MYOC protein. In addition, MYOC protein is reported to be cleaved at amino acids 226/227 [23] and is also reported to be aggregation-prone [24]. All these factors complicate endeavors to obtain purified full-length (FL) MYOC protein for functional studies and protein structure analysis.

To date, only small portions of the MYOC N-terminal have been reported to be purified from *E. coli* [25] while the MYOC C-terminal OLF-domain has been purified from *E. coli* and its structure solved [7]. As we desired FL MYOC protein for functional studies and screening endeavors, we began MYOC protein purification efforts. Herein, we present two methods by which to obtain purified FL MYOC protein from mammalian cell lines. With the first method, we identified a cell line (HeLa S3) that permits sufficient quantities of FL MYOC protein to be collected from cell media. In the second method, we identified the MYOC cleavage site in 293 cells and created plasmids which had point mutations in the cleavage site thus allowing FL MYOC protein to be purified. We have found that the majority of purified FL MYOC protein is in a dimer form and we propose a modeled structure for dimeric MYOC. The ability to isolate FL MYOC will help scientists identify MYOC binding partners and this will assist in efforts to understand functional properties of MYOC thereby enabling the determination of the role of this unique protein in health and disease.

2. Methods

2.1. Human samples

Human samples were obtained with consent from regional donor eye banks. Aqueous humor (AH) was collected from cadaver donor eyes (< 12 h post-mortem) and stored at −80 °C until use. Medical histories provided patient information. All samples we utilized were from donors of a similar elderly age. Note that none of the donors carried a *MYOC* mutation.

2.2. Gel LC MS-MS analysis of human aqueous humor

Upon separation of normal and glaucoma human AH samples by SDS-PAGE and staining with SimplyBlue (Invitrogen, LC6065), protein bands were excised from gels and trypsin digested. Trypsin digestion was performed according to standard procedures [26]. Briefly, the gel pieces were dehydrated with acetonitrile (Thermo Fisher Scientific) and rehydrated with 100 mM NH_4HCO_3 buffer containing 10 mM dithiothreitol (Thermo Fisher Scientific) to reduce disulfide bonds. Gel pieces were treated with 100 mM NH_4HCO_3 buffer containing 20 mM iodoacetamide (Thermo Fisher Scientific) to alkylate cysteines. After two rounds of dehydration with acetronitrile and rehydration with 100 mM NH_4HCO_3 buffer, the dried gel pieces were rehydrated with 20 ng/μL trypsin sequencing grade (Roche) in 50 mM NH_4HCO_3 buffer and incubated over-night at 37 °C. Peptides were extracted with 75% acetonitrile/0.1% formic acid and dried in a vacuum centrifuge. Samples were resuspended in 5% acetonitrile/0.1% formic acid and introduced to an Orbitrap Fusion Lumos (Thermo Fisher Scientific) using an EASY nano-LC1200 system (Thermo Fisher Scientific) with an analytical column packed with a 0.075 × 200 mm ReproSil-Pur C18-AQ, 3 μm (Dr. Maisch, Ammerbuch, Germany).

Mass spectrometry (MS) and tandem MS/MS spectra were performed on an Orbitrap Fusion Lumos mass spectrometer operated on data-dependent acquisition mode. Survey MS1 scans were acquired in the Orbitrap using a 350–1400 m/z range at 120,000 resolution. The most intense ions per survey scan (top speed mode), rising above threshold, were selected for HCD fragmentation, and the resulting fragments were analyzed in the Orbitrap. Proteome Discoverer software suite (v2.1, Thermo Fisher Scientific) was used for peptide identification. The data were searched against the UniProt rat database (version Nov 2015). At the MS1 level, a precursor ion mass tolerance of 10 ppm was used, and up to three missed cleavages were allowed. The fragment ion mass tolerance was set to 20mmu for the Orbitrap MS/MS detection methods. Oxidation of methionine was defined as a variable modification and carbamidomethylation on cysteines was defined as a fixed modification. False discovery rate (FDR) in peptide identification was limited to a maximum of 0.01 by using a decoy database. Abundance data were retrieved from the “Precursor ion area detector” node from Proteome Discoverer (v2.0) using 2 ppm mass tolerance for the peptide extracted ion current (XIC).

2.3. Cell lines and transfection methods

In this report, numerous immortalized mammalian cell lines, including 293 variants, were utilized.

2.3.1. NTM5

An immortalized normal trabecular meshwork cell line 5 (NTM5) was used and this cell line has previously been described [27]. MYOC is a protein whose expression in primary cells declines with culture time [22] and NTM5 cells no longer express MYOC protein nor is MYOC expression in these cells influenced by dexamethasone treatment. NTM5 cells were grown in 10 cm culture dishes in a 37 °C incubator with 90% relative humidity and 5% CO_2. Cell media was DMEM (Gibco, 11995-065) supplemented with 10% FBS (Gibco, 10082147) and 1% P/S (Gibco, 15140-122). NTM5 cells at 70–80% confluence were transiently-transfected with plasmids (6 μg total cDNA per 10 cm plate) using FuGENE6 transfection reagent (Promega, E2691). FuGENE6 was utilized at a 5:1 ratio with the cDNA.

2.3.2. COS7 and 293 cells

Cell media for both COS7 cells and 293 cells was DMEM (Gibco, 11995-065) supplemented with 10% FBS (Gibco, 10082147) and 1% P/S (Gibco, 15140-122).