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The identification of myocilin-associated proteins in the human trabecular meshwork

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Abstract

Myocilin forms high molecular weight complexes in vivo presumably due to interaction with itself and other myocilin binding proteins. To identify myocilin interacting proteins, yeast 2-hybrid analysis was performed on $>1\times10^6$ human trabecular meshwork cDNA clones. Coimmunoprecipitation and Far Western analysis were also performed on cell lysates obtained from fresh human trabecular meshworks or cultured human monolayer trabecular cell lines. Among the different methods, 46 candidate myocilin-associated proteins were identified, including molecules associated with the extracellular matrix, cytoskeleton, signaling, and metabolism. The most consistent interaction was myocilin–myocilin binding. Yeast-2 hybrid and Far Western analysis also found an association between myocilin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). None of the other candidate myocilin interacting proteins were identified in more than one method. Characterization of these potential interacting proteins may help to better understand the function of myocilin in the trabecular meshwork and aqueous outflow pathway.

Keywords: myocilin; myocilin-associated proteins; trabecular meshwork; TIGR

1. Introduction

Myocilin is present in aqueous humor in complexes with molecular weights >120 kDa (Rao et al., 2000; Russell et al., 2001; Fautsch and Johnson, 2001; Fautsch et al., 2004). Because the monomeric form of myocilin is 53–57 kDa, it is believed that myocilin forms these complexes with itself and other associated proteins. The primary structure of myocilin contains an N-terminal leucine zipper that is important for binding with itself (Fautsch and Johnson, 2001). In addition, myocilin contains five cysteines that through disulfide bond formation, may form intramolecular or intermolecular disulfide bonds (Fautsch et al., 2004).

A logical step in understanding the role of myocilin in aqueous outflow is to determine its physiologic location within the trabecular meshwork. Immunohistochemical studies using light and electron microscopy suggest myocilin is present in both the cells and the extracellular matrix of the meshwork, associating with mitochondria, intercellular junctions, the cytoskeleton, and the amorphous-like basement membrane (Lutjen-Drecoll et al., 1998; Ueda et al., 2000, 2002; Ueda and Yue, 2003).

Molecularly, several extracellular matrix proteins have been reported as myocilin binding molecules. These studies identified fibronectin (Filla et al., 2002; Ueda et al., 2002), laminin (Ueda et al., 2002), decorin (Ueda et al., 2002), collagen I (Ueda et al., 2002), and collagen III (Ueda et al., 2002). Optimedin, a secreted olfactomedin-related protein, has also been identified as a potential myocilin binding protein (Torrado et al., 2002). Intracellularly, myosin regulatory light chain has been shown to interact with myocilin, however this interaction occurred with a truncated myocilin protein (Wentz-Hunter et al., 2002).

In an attempt to identify candidate myocilin binding partners and to verify the previously reported interacting proteins, we analyzed myocilin complexes in fresh human trabecular meshwork and cultured monolayer trabecular cells. Proteins associated with the extracellular matrix, cytoskeleton, signaling, and metabolism were identified.

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2. Methods

2.1. Yeast two-hybrid

The yeast two-hybrid system is a transcriptional assay performed in live yeast to determine protein-protein interactions. In brief, the yeast two-hybrid system uses the finding that gene transcription factors contain two domains: a DNA-binding domain (BD) and an activation domain (AD). While generally found on the same protein, these domains can remain functional even if placed on separate proteins, provided that these proteins interact with each other and thus bring the attached BD and AD near each other. The yeast two-hybrid system exploits this phenomenon by fusing a BD domain to one protein (myocilin) and an AD domain to another protein (trabecular meshwork cDNA library). Both proteins with their coupled BD or AD are expressed in yeast. If interaction of the proteins occurs, the BD and the AD will activate transcription of amino acid reporter genes that allow yeast to grow on selective media.

A trabecular meshwork library was made from the pooling of nine trabecular meshworks isolated from five pairs of human donor eyes (77 ± 2 years; obtained within 12 hr of death; histology was analyzed by light microscopy to verify healthy meshworks). Total RNA was isolated and converted to double-stranded cDNA using a commercial kit (BD Biosciences, Palo Alto, CA). cDNA was inserted into yeast expression vector pGADT7-AD and transformed into AH109 competent yeast. Transformed yeast were plated onto minimal media agar plates (-leucine) and grown at 30 °C for 4 days. Yeast containing trabecular meshwork cDNA were collected, mixed, and aliquoted.

Myocilin expression in yeast was accomplished by inserting myocilin cDNA (nucleotides 133–1551; does not contain nucleotides coding for signal peptide sequence) into yeast expression vector pGBKT7-BD (BD Biosciences) and transforming it into competent Y187 yeast. Y187 yeast were plated on minimal media agar plates (-tryptophan) and positive colonies selected. Isolation of plasmid DNA followed by sequence analysis verified the correct insertion and sequence of myocilin.

Y187 yeast (containing pGBKT7-myocilin) and AH109 yeast (contains trabecular meshwork cDNA clones) were mated by mixing together the two yeast strains at 30 °C overnight. The yeast matings were spread onto minimal media agar plates (-adenine, -histidine, -leucine, -tryptophan). Yeast containing both pGBKT7-myocilin and pGADT7-cDNA clones formed colonies on minimal media plates (6 days of growth at 30 °C). Yeast colonies present after 6 days of growth at 30 °C were isolated. Plasmid DNA was extracted from each independent yeast colony and transformed into DH5α bacteria. Bacteria were screened for growth on ampicillin plates. Positive bacterial colonies were expanded and plasmid DNA was extracted. DNA sequence analysis was performed to determine identity of the trabecular meshwork cDNA clone. In two independent yeast matings, over 1×10^6 transformed colonies were screened.

2.2. Coimmunoprecipitation

Three experiments were performed, using similar methods. The initial two experiments were done with seven human trabecular meshworks each to optimize conditions. The final experiment was done with 51 human trabecular meshworks from 26 donors (70.3 ± 17.4 years; mean ± S.D.). Meshworks were placed in 0.7 ml of modified RIPA buffer [50 mM Tris pH 8.0, 1% Triton, 0.1% SDS, 137 mM NaCl, 3 mM KC1, 8 mM Na_2HPO_4, l mM KH_2P0_4, 75 μg/ml PMSF, and TM protease inhibitors (Roche Applied Sciences, Indianapolis, IN)], homogenized, and incubated at 4 °C for 30 min. Sample was centrifuged at 13 000 × *g* for 15 min and supernate was collected and placed at 4 °C. Addition of 0.3 ml of RIPA buffer to trabecular meshworks followed by re-homogenization and incubation at 4 °C was repeated two other times. The supernate from the three homogenizations were combined. A Bradford protein assay was performed on trabecular meshwork lysate to determine protein concentration (Bio-Rad, Hercules, CA).

Affinity purified anti-myocilin antibodies (Fautsch et al., 2000) were attached to Carbolink™ beaded agarose (Pierce, Rockford, IL) and added to 2.4 mg of trabecular meshwork lysate. Sample was placed shaking at room temperature for 90 min followed by an overnight incubation at 4 °C. A control reaction was performed with Carbolink™ beaded agarose (no antibody attached) and incubated with trabecular meshwork lysate. Following overnight incubations, Carbolink™ beaded agarose was pelleted by centrifugation at 13 000 × *g* for 1 min. The supernate was removed and the pellet was resuspended in 500 μl of phosphate-buffered saline. Carbolink™ beaded agarose was transferred to Handee Spin Cup Columns (Pierce Biotechnology, Rockford, IL). Columns were centrifuged at 13 000 × *g* for 1 min. The supernate was discarded and Carbolink™ beaded agarose was resuspended in 500 μl of phosphate-buffered saline, incubated at room temperature for 5 min, and centrifuged at 13 000 × *g* for 1 min. Wash procedure was repeated a total of six times.

Myocilin and myocilin-associated proteins were eluted from the Carbolink™ beaded agarose with 0.1% SDS and twice with 0.5% SDS. Elutions of myocilin and control immunoprecipitations (30 μl) were placed in Laemmli sample buffer. Samples were boiled and separated on 4–15% SDS-PAGE gels (Bio-Rad). Gels were silver-stained. Proteins present in the myocilin immunoprecipitation lane, but not in the control, were excised and sequenced as previously described (Fautsch et al., 2005).

2.3. Far Western

This technique modifies the traditional Western by using two probes in succession. In our two studies, the first probe was recombinant myocilin purified from the media of cultured trabecular meshwork cells overexpressing myocilin (Fautsch et al., in press). The recombinant myocilin contains a V5 and six-histidine epitope tag in the C-terminus. As a control, an additional purification was performed on media isolated from the parental human trabecular cell line. The second probe was