Protein Interactions with Myocilin

Kelly Wentz-Hunter, Jun Ueda, and Beatrice Y. J. T. Yue

PURPOSE. To identify factors that interact in vivo with myocilin, a glaucoma gene product.

METHODS. The yeast two-hybrid system with myocilin as the bait and a human skeletal muscle cDNA library as the prey was used to identify potential factors that interact with myocilin. Interactions were also examined in bovine trabecular meshwork (TM) cells through a mammalian two-hybrid system. Biochemical communoprecipitation from both human TM cell lysate and in vitro translated proteins was also used to confirm results obtained from yeast analysis.

RESULTS. Twenty positive clones isolated through yeast two-hybrid screening were deemed potential myocilin partners. Sequence analysis determined that two of them encoded for myocilin from amino acids 64 to 268. Myocilin was also found to interact with a component of the myosin motor protein, myosin regulatory light chain (RLC). The myocilin–myocilin and myocilin–RLC interactions revealed by the yeast system were further confirmed and demonstrated in cultured TM cells, by means of a mammalian two-hybrid system, and through biochemical communoprecipitation, subcellular fractionation, immunofluorescence, and immunogold double labeling.

CONCLUSIONS. These results indicate that myocilin can form homomultimers in vivo, independent of the olfactomedin-like domain. Further analysis established that the leucine zipper motif of myocilin may be necessary for the myocilin–RLC interaction. The interaction of myocilin with RLC, a component of the myosin motor protein complex, implies a role for myocilin in the actomyosin system, linking in turn this novel protein to functional status of the TM. (Invest Ophthalmol Vis Sci. 2002;43:176–182)

Glaucoma, a leading cause of blindness in the United States, is a disease generally characterized by elevation of the intraocular pressure, damage to the optic nerve head, and eventual visual loss. Primary open-angle glaucoma (POAG), the most common form of glaucoma, is genetically heterogeneous, with varying modes of inheritance. One of the foremost risk factors of POAG is elevated intraocular pressure that results from abnormalities in the aqueous humor outflow pathway. The trabecular meshwork (TM) is a major site regulating the normal bulk flow of the aqueous humor. Cells in the TM, with an endothelial cell–like morphology and lining property, are believed to be vital for normal maintenance of the outflow pathway. Dysfunction or alteration of TM cell activities may be responsible for the development of glaucoma.

Myocilin, also called trabecular meshwork-inducible glucocorticoid response protein (TIGR), has been directly linked to open-angle glaucomas. Multiple mutations were identified in a number of families. TIGR was initially identified as a major 55-kDa protein secreted into the media of TM cultures after induction with glucocorticoids, such as dexamethasone. This gene was also identified by Kubota et al. in the retina and was termed myocilin for its sequence similarity to myosin. It is sometimes also referred to as MYOC/TIGR or TIGR/MYOC.

Analyses of the genomic sequence of myocilin have identified an N-terminal myosin-like domain (corresponding to amino acids 72–179) that includes a leucine zipper motif and a C-terminal olfactomedin-like domain (amino acids 324–502). Between the two domains is an undefined linker region. Myocilin is also known to exist in 66- and 57-kDa isoforms.

The myocilin transcript has been detected in ocular tissues including the retina, TM, ciliary body, iris, and optic nerve head and in nonocular tissues such as skeletal muscle and heart. Recent studies have focused on a greater understanding of the localization of the myocilin protein as well as insight into the control of its expression through regulatory promoter elements. Data from our laboratory using immunogold electron microscopy (EM) have revealed that myocilin is localized to both intracellular and extracellular sites in the TM. Intracellularly, myocilin was found to be associated with mitochondria, vesicles, intermediate filaments, and actin stress fibers. Extracellularly, myocilin is found in association with extracellular matrices.

Despite these efforts, the exact nature of myocilin and its function remain elusive. This information is certainly a prerequisite for the uncovering of how overexpression or variant forms of myocilin would produce disease in open-angle glaucomas. We herein describe a study conducted to identify factors interacting with myocilin. Techniques including yeast and mammalian two-hybrid screening, communoprecipitation, subcellular fractionation, immunofluorescence, and immunogold double labeling were incorporated. Our results indicate that myosin regulatory light chain (RLC) is an interacting partner of myocilin and shed light on a possible functional role of myocilin through its link to the myosin motor protein complex.

MATERIALS AND METHODS

Yeast Two-Hybrid Analysis

The screening for protein interactions with myocilin was performed with a human skeletal muscle cDNA library (Clontech, Palo Alto, CA). Myocilin full-length and myocilin 64-504 (with amino acids 1-63 deleted) were amplified by PCR. Primers were: myoc 5′-GGAATTCAT-GAGGTTCCTTCTGAGGCGTGGTCG-3′; myoc 3′-GGAATTCAGCT-TGGGAGGTTTTTACACATGG-3′; and myoc Δ1-63 5′-GGAATTCACAGGAGGCGATGTCG-3′. Myocilin was amplified from a human TM cDNA library and sequenced by the DNA sequencing facility at the University of Chicago. Myocilin constructs were subcloned using the EcoRI site downstream of the GAL4 DNA binding
domain in pAS2-1. Yeast transformations were performed simultaneously. All clones that grew on selective minimal media and were positive for β-galactosidase were subject to controls to identify false-positives. Firstly, cDNA prey plasmids were isolated and transformed into Y190 alone and assayed for β-galactosidase activity to detect intrinsic DNA-binding ability. Second, the cDNA plasmids were transformed into Y190 with pAS2-1 without insert or with pVA3, the murine p53 gene subcloned into pAS2-1, to rule out the possibility of nonspecific protein interactions. Plasmid DNA from the true positive clones was sequenced and analyzed by BLAST.

**Cell Cultures**

Normal human eyes were obtained from either the Illinois Eye Bank or the National Disease Research Interchange. The procurement of tissue was approved by the Institutional Review Committee at the University of Illinois at Chicago. Fresh bovine eyes were obtained from Aurora Packing Company (Aurora, IL). TM tissues were dissected and cultured as previously described.

**Mammalian Two-Hybrid System**

A mammalian two-hybrid system (Clontech) was used to verify protein–protein interactions initially identified in the yeast screening. The myocilin 64–504 was subcloned into either the pM or pVP16 cloning vector to generate myocilin bait or prey fusion proteins, respectively. For immunoprecipitation, a kit was used (Matchmaker Co-IP kit; Clontech). Briefly, T7 promoters and either c-myc or hemagglutinin (HA) epitope tags were incorporated respectively by PCR into myocilin 9

**TABLE 1.** CAT Activity from Mammalian Two-Hybrid in Bovine TM Cells

<table>
<thead>
<tr>
<th>Bait</th>
<th>Prey</th>
<th>CAT Activity</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myocilin (64–504)</td>
<td>Myocilin (64–504)</td>
<td>0.289 ± 0.018</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Myocilin (64–504)</td>
<td>RLC</td>
<td>0.315 ± 0.011</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>DNA-BD</td>
<td>AD</td>
<td>0.048 ± 0.005</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD, n = 3. OD, optical density; DNA-BD, DNA-binding domain; AD, activation domain.

**Coimmunoprecipitation**

For immunoprecipitation, a kit was used (Matchmaker Co-IP kit; Clontech). Briefly, T7 promoters and either c-myc or hemagglutinin (HA) epitope tags were incorporated respectively by PCR into myocilin 64–504 and RLC cDNAs using the primers provided. For further myocilin deletions, primers were: myoc 226 TCTAGTTA-3; myoc 109 CCTGGGACTGTTCCAGGCTTATT-3; and myoc 310 5′-GGAAGCCTCCGGCAGGACCATCCTGTAACCT-3′. The products were in vitro transcribed and translated using a TNT T7-coupled reticulocyte lysate system (Promega, Madison, WI) and 35S-labeled methionine (Amersham, Piscataway, NJ). For coimmunoprecipitation, the translated c-myc-myocilin 64–504 and HA-RLC were mixed at 35°C for 1 hour. The mixture was then incubated with co-IP buffer, protein Aagarose beads and either monoclonal c-myc or polyclonal HA antibody at 4°C for 2 hours. After washing, the beads were resuspended in SDS-loading dye. The proteins recovered were resolved on a 4% to 15% linear gradient SDS-polyacrylamide gel. The gel was then fixed and treated with FluororEnhance (Research Products Inc., Mount Prospect, IL). The radioactive protein bands were visualized using a phosphorescence imager (Cyclone Storage Phosphor System; Packard, Meriden, CT). For negative control experiments, either the in vitro translated products were incubated in the presence of exogenous protein, or the antibody was replaced with a nontagged antibody.

Coimmunoprecipitation was also completed using human TM cell lysates. Cultured TM cells were lysed on ice in 10 mM Tris-HCl (pH 8.0) 150 mM NaCl, 0.5% NP-40, 2 mM phenylmethylsulfonyl fluoride, and 1× cocktail protease inhibitors (Roche Molecular Biochemicals). Nuclei and cellular debris were pelleted, and the lysate collected was precleared with excess goat anti-rabbit IgG-conjugated affinity gel (ICN/Cappel, Costa Mesa, CA). Proteins were immunoprecipitated with either anti-myocilin or preimmune rabbit serum. Anti-myocilin was raised in rabbits against a synthetic peptide corresponding to amino acids 33-43 (RTAQLRKANDQ). The peptide was coupled to keyhole-limpet hemocyanin through a C-terminal cysteine residue not present in myocilin. The synthetic peptide was made, and the antibody was raised and affinity purified by Alpha Diagnostic International (San Antonio, TX). The antibody specificity was verified by Western blot analysis, as previously described for another anti-myocilin peptide antibody.

The antibody–protein complex precipitated with the affinity gel was resuspended in reducing sample buffer. Proteins were separated on a 10% SDS-PAGE and transferred to nitrocellulose (Protran, Mid west Scientific, St. Louis, MO). The membrane was probed with anti-RLC23 (1:1000, Sigma, St. Louis, MO) and horseradish peroxidase-conjugated goat anti-mouse IgM (1:10,000, ICN/Cappel). Protein bands were visualized with enhanced chemiluminescence (Amersham, Arlington Heights, IL). The absorbance at 600 nm was read on a microplate reader. Interactions between the binding and activation domain proteins would promote the CAT gene expression, resulting in enhanced CAT activity. Student’s t-tests were used to determine the statistical significance of the data.

**FIGURE 1.** Coimmunoprecipitation of myocilin and RLC. c-Myc–myocilin 64–504 and HA-RLC were in vitro translated in the presence of 35S-methionine. c-Myc–myocilin 64–504 and HA-RLC were mixed together and immunoprecipitated with either anti-c-myc (lane 1) or anti-HA antibody (lane 2). c-Myc–myocilin alone was also immunoprecipitated with anti-HA (lane 3) and HA-RLC with anti-c-myc (lane 4) as negative controls. Arrow: RLC, arrowheads: myocilin.
subcellular fractionation of human TM cell extracts. Proteins fractionated were immunoblotted with anti-myocilin (a) and anti-RLC (b) antibodies. Only fractions 10 to 17 are shown.

**Subcellular Fractionation of Human TM Cells**

Subcellular fractionation was performed using a density gradient solution (Optiprep; Accurate Chemical and Scientific Co., Westbury, NY) and ultracentrifugation. A discontinuous gradient was prepared using 30%, 25%, 20%, 15%, and 10% solution (Optiprep). The gradient was allowed to equilibrate vertically at room temperature for 30 minutes. Human TM cells, after a wash in PBS, were harvested in homogenization buffer (0.25 M sucrose, 10 mM HEPES-NaOH [pH 7.4], 1 mM EDTA) and broken open by repeated strokes in a homogenizer (Dounce; Bellco Glass Co., Vineland, NJ). Cell debris and nuclei were then pelleted by centrifugation at 1000 g for 10 minutes. The post-nuclear supernatant was overlaid onto the discontinuous gradient and centrifuged at 100,000 g for 3 hours at 4°C. Equal fractions were collected from the top of the gradient. Proteins were separated on 10% SDS-PAGE and immunoblotted with anti-myocilin (1:2000) or anti-RLC (1:1000), as for coimmunoprecipitation.

**Immunofluorescence**

Immunofluorescence staining was completed with a kit (Tyramide Signal Amplification-Direct Kit; NEN-Life Science, Boston, MA). Human TM cells were fixed in paraformaldehyde-lysine-phosphate buffer (pH 7.4) and permeabilized in 0.2% Triton X-100. Primary antibodies were anti-myocilin (1:200) and anti-RLC (1:100). Secondary antibodies were horseradish peroxidase-conjugated goat anti-rabbit IgG and Cy3-conjugated goat anti-mouse IgM (1:200, both from Jackson ImmunoResearch, West Grove, PA). Cells were subsequently incubated with the FITC-tyramide solution (1:50 diluted in amplification buffer) for 10 minutes, washed, and mounted (Crystal Mount; Biomedica Corp., Foster City, CA). The staining was visualized by confocal microscope (model 100M; Carl Zeiss Jenova GmbH, Jena, Germany).

**Immunogold EM**

Human TM cells were fixed and processed as previously described.26 Primary antibodies were anti-myocilin (1:200) and anti-RLC (1:50; Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies were 12-nm colloidal gold-conjugated goat anti-rabbit IgG and 6-nm gold sheep anti-goat IgG (1:30; Jackson Immunoresearch). After washing, the samples were counterstained with uranyl acetate and examined under a transmission electron microscope (JEM-1220; JEOL, Tokyo, Japan).

**RESULTS**

**Yeast Two-Hybrid Screening**

Yeast two-hybrid screening25 was performed to identify interactions between myocilin and other proteins. Initially, the myocilin full-length cDNA was used as the bait and a human skeletal muscle cDNA library as the prey. After screening more than $10^6$ independent transformants, however, we were unable to identify any interacting clones from the skeletal muscle library. The full-length bait protein structure may not allow accessibility for binding with myocilin, or the bait protein could have been cleaved off at the putative cleavage site at amino acid 32. Therefore, a fragment containing the 1-63 amino acid deletion, myocilin 64–504, that does not include the cleavage site, was constructed as the bait. Approximately $11 \times 10^6$ independent transformants of the human skeletal muscle library were subsequently screened. Twenty-six transformants that showed both growth on histidine minus medium and production of β-galactosidase were identified.

A series of control experiments, detailed in experimental procedures, was completed to rule out false-positives. After the elimination process, 20 of the 26 cDNA clones were judged to be true-positives. These clones were isolated and sequenced using a GAL4 activation domain specific primer. Sequence analyses determined that the 20 clones encoded only 11 distinct entities. Comparison with the GenBank database revealed that four of these were novel sequences (GenBank is provided by the National Center for Biotechnology Information, Bethesda, MD, and is available at http://www.ncbi.nlm.nih.gov/genbank/).

The sequence of two of the cDNA library clones was identical with portions of myocilin. The clones began at amino acid...
64, located at the beginning of the myosin-like domain with the sequence homologous with myocilin up to amino acid 268 in the linker region.

Sequence analysis of another clone isolated twice from the library screening identified ventricular RLC 26 (GenBank accession number NM 00432) as a myocilin-interacting molecule. The cDNA library clone encoded the entire RLC cDNA as well as 33 nucleotides of the 5' untranslated region. Although the insert contained the 5' untranslated region, the clone remained in-frame and encoded the complete RLC protein, starting at the first methionine codon.

**Mammalian Two-Hybrid Analysis**

To verify that the myocilin–myocilin and myocilin–RLC interactions discovered in the yeast system also took place in a mammalian environment, bovine TM cells were cotransfected with myocilin 64-504 subcloned into the mammalian expression vector pM serving as the bait, myocilin 64-504 or RLC subcloned into pVP16 as the prey, and a reporter vector pG5CAT. The bait and prey vectors without inserts were used as negative controls. As shown in Table 1, the resultant CAT activity in transfected cells, with myocilin 64-504 as the bait and either myocilin 64-504 or RLC as the prey, was approximately six times higher ($P < 0.0001$) than the negative control values, reaffirming the bait–prey interactions in mammalian cells.

**Biochemical Confirmation of Myocilin and RLC Interaction**

Myocilin 64-504 and RLC were in vitro translated in the presence of $^{35}$S-methionine to produce c-myc-tagged myocilin 64-504 and HA-tagged RLC. When allowed to interact, c-myc-myocilin 64-504 or HA-RLC proteins coimmunoprecipitated by either c-myc (Fig. 1, lane 1) or HA (Fig. 1, lane 2) antibody. In negative controls, when the tagged proteins were incubated with the opposite antibody, no nonspecific precipitation occurred (Fig. 1, lanes 3 and 4). Immunoprecipitation of c-myc-myocilin 64-504 with anti-c-myc yield a band of 50-kDa and higher molecular weight bands at approximately 100 and 200 kDa under nonreduced conditions (data not shown).

The interaction of myocilin and RLC appeared to take place in human TM cells as well. Protein complexes were immuno-
precipitated from lysates of cultured human cells with either anti-myocilin or preimmune serum. The immunoprecipitated protein complexes were run on SDS-PAGE, and Western blot analysis was performed using anti-RLC. The sample immunoprecipitated with anti-myocilin pulled down a 20-kDa RLC band (Fig. 2A, lane 1). This was not observed when preimmune serum was used (Fig. 2A, lane 2).

Subcellular fractionation experiments with human TM cell extracts also confirmed an association between myocilin and RLC. After subcellular fractionation, 17 equal fractions were collected from the top of the gradient. The fractions were subjected to SDS-PAGE and Western blot analysis for myocilin and RLC (Fig. 2B). Myocilin was detected in fractions 12 to 17 and RLC was in fractions 13 to 15, indicating that the two proteins sedimented in similar subcellular compartments.

**Colocalization of Myocilin and RLC**

Immunofluorescence staining (Fig. 3) of human TM cells with anti-myocilin demonstrated staining in the perinuclear region that extended toward peripheral regions. RLC immunostaining revealed a fibrillar distribution pattern. Double staining with both anti-myocilin and anti-RLC showed overlap of distribution, particularly on fibrillar structures. Immunogold EM localized RLC to actin stress fibers and cytoplasmic filaments in human TM cells. Double labeling with different sized colloidal gold particles further demonstrated the presence of both myocilin and RLC on actin stress fibers (Fig. 4). Colocalization in close proximity was evident in scores of areas.

**Domain of Interaction between Myocilin and RLC**

To map further the domain of myocilin necessary for its interaction with RLC, three deletion constructs were prepared (Fig. 5A). One contained the entire myosin-like domain (amino acids 64–226), one had only part of the myosin-like domain (64–109), and the third contained the olfactomedin-like domain (310–504). These myocilin constructs were translated in vitro, and coimmunoprecipitation experiments showed that the myocilin construct 64–226 retained the RLC-interacting capability, whereas the other two did not (Fig. 5B). Negative control experiments yielded no bands (data not shown).

**DISCUSSION**

More than 50 mutations have been identified in the myocilin gene since the first report of its linkage to open-angle glaucomas. Although the defects associated with mutations remain to be elucidated, recent mutation studies have led to the recognition that factors in addition to mutations may be involved in the development of glaucoma and that protein–protein interactions of myocilin may be of paramount importance.

In the present investigation, we screened a human skeletal muscle cDNA library using the yeast two-hybrid system to identify interacting partners of myocilin. After screening of 11,310 transformants, 11 distinct clones were identified. One of them encodes for a portion of the myocilin protein from amino acids 64–268. The current results thus indicate that myocilin can complex with itself to form a homodimer or oligomer in vivo. Previously, it was suggested, based on molecular weights of protein bands in Western blots that myocilin multimers could be formed in vitro. In our investigation, using both the yeast and bovine TM cells, results suggest confirmation of such a formation in vivo. In addition, from the sequence of the cDNA library clone, we conclude that the N-terminal and the olfactomedin-like domain are not essential for the multimerization.

Another clone isolated from the yeast two-hybrid screen encodes the full-length RLC and 33 amino acids of the 5′ untranslated region. The interaction between myocilin and
RLC was confirmed in bovine TM cells using the mammalian two-hybrid system and by biochemical coimmunoprecipitation. Myocilin and RLC were found to be in similar compartments in human TM cell extracts. In addition, colocalization was observed by immunofluorescence and immunogold double labeling.

The region in myocilin for its interaction with RLC appears to be located between amino acids 109 and 226, encompassing the leucine zippers (amino acids 117–169) within the myosin-like domain (amino acids 72–179). This region of myocilin is highly conserved with 65% amino acid identity across species, suggesting a possible functional importance. Leucine zipper motifs, consisting of heptad repeats of leucine residues that form a coiled-coil structure, have been shown to be essential for protein-protein interactions. Well-known examples include transcription factors such as c-fos and c-jun that form homo- and heterotypic dimers through leucine zipper motifs for their functions.

This myosin light chain is a component of the myosin heterohexamer along with two copies each of the myosin heavy chain and a single light chain. RLC interacts with myosin heavy chain through the IQ motif (IQXXXRGXXXR).32 This interaction does not involve the heavy chain leucine zipper domain, where it bears high sequence homology to myocilin. The nature of the RLC-myocilin interaction therefore may be distinct from that of RLC-myosin heavy chain.

The actin-based myosin motor protein complex is critical in many cellular processes in both muscle and nonmuscle cells. The importance of a functional actomyosin system has been demonstrated in the TM system. Recent studies both in organ culture5,5 and live monkeys8,8 have shown that a myosin light chain kinase inhibitor, H-7, inhibits actomyosin-based contractility in TM cells, causing disruption of actin filament organization with resultant changes in the outflow facility and intraocular pressure. Taken together, we hypothesize that the interaction between myocilin and RLC may be functionally relevant. Through RLC, myocilin may be linked to the actomyosin system in the TM and, in turn, take part in aqueous humor outflow regulation. Mutations or overexpression of myocilin may alter the RLC-myosin heavy chain dynamics and the activity of the actomyosin motor complex in TM cells. Such a possibility provides a means by which abnormalities in myocilin may affect the functional status of TM cells and lead to glaucoma. Although additional work is warranted to establish the precise role of myocilin in the myosin motor function and actin cytoskeleton organization, the current finding distinctly implicates myocilin as a component of these processes.

Acknowledgments

The authors thank E. Lillian Cheng for cell culture work and Kira Lathrop for expert imaging.

References


