Molecular Chaperone Function for Myocilin

Ann Marie Anderssohn,^{1,2} Kalani Cox,^{1,2} Kevin O'Malley,² Scott Dees,² Mojgan Hosseini,^{1,3} Lacey Boren,² Anthony Wagner,² John M. Bradley,¹ Mary J. Kelley,¹ and Ted S. Acott¹

PURPOSE. Myocilin is thought to be a stress response protein, but its exact molecular functions have not been established. Studies were conducted to see whether myocilin can act as a general molecular chaperone.

METHODS. Myocilin was isolated and purified from porcine trabecular meshwork (TM) cell culture media. Its ability to protect citrate synthase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the restriction endonuclease *DrdI* from thermal inactivation was evaluated. Light scattering was used to evaluate thermally induced aggregation of citrate synthase. Myocilin induction was assessed after exposure of TM cells to several types of stress treatments.

RESULTS. Levels of extracellular myocilin expressed by TM cells were increased in response to mechanical stretch, heat shock, TNF α , or IL-1 α . Myocilin protected citrate synthase activity against thermal inactivation for 5 minutes at 55°C in a concentration-dependent manner, with nearly full protection of 1.5 μ M citrate synthase in the presence of 650 nM myocilin. Myocilin significantly reduced thermal aggregation of citrate synthase to levels 36% to 44% of control levels. Myocilin also protected GAPDH from thermal inactivation for 10 minutes at 45°C. Myocilin at 18 nM was more effective than 1 μ M bovine serum albumin at protecting *DrdI* from thermal inactivation.

CONCLUSIONS. Myocilin is induced in response to several cellular stresses and displays general molecular chaperone activity by protecting *DrdI*, citrate synthase, and GAPDH from thermal inactivation. Myocilin also suppresses the thermal aggregation of citrate synthase. One function of myocilin may be to serve as a molecular chaperone. (*Invest Ophthalmol Vis Sci.* 2011;52:7548–7555) DOI:10.1167/iovs.11-7723

Glaucoma, which affects approximately 70 million people, is a major cause of blindness throughout the world.¹ The most common form, primary open-angle glaucoma (POAG), is typically associated with elevated intraocular pressure, loss of peripheral vision, and accompanying damage to the optic nerve.² Myocilin mutations have been associated with juvenile

and early onset POAG and a subset of adult POAG.^{3–6} Polansky et al.^{7,8} identified and characterized a TM-inducible glucocorticoid response protein, which is also known as myocilin.⁹ *MYOC*, the myocilin gene, codes for a 504-aa glycoprotein with homology to olfactomedin, a mucoid protein found in neuro-epithelium.^{8,10,11} It includes structural motifs for both N- and O-linked glycosylation, hyaluronan binding, and a myosin-like leucine zipper.⁸ Myocilin is widely distributed in ocular tissues,^{10,12–16} and its mRNA is also expressed in various nonocular tissues, including the heart, skeletal muscle, kidney, and peripheral nerves.^{8,10,17–21} Both intracellular nonglycosylated and extracellular glycosylated and nonglycosylated forms of myocilin have been identified.^{8,10} On gels, the primary band runs as a doublet of approximately 55 and 57 kDa, with a 66-kDa band apparent in some cases. Myocilin associations have been reported with mitochondria,^{22–24} exosome-like vesicles,^{25,26} and components of the cytoskeleton and extracellular matrix.^{14,27–33} Myocilin interactions have also been demonstrated with a number of molecules associated with cell signaling and metabolism, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH),³⁴ and optomedin, which is itself an olfactomedin-related protein.³⁵ To date, no clear molecular function for myocilin has been established,^{10,36} and myocilin double knockout or overexpressing mice show no clear phenotype.^{37–40} Perfusion studies with recombinant myocilin have demonstrated both increases and decreases in outflow facility, depending on conditions.⁴¹ In addition, studies of the glaucoma-related myocilin mutations suggest an involvement of the misfolded or unfolded protein response⁴² in disease etiology.^{10,43–48} Recent studies⁴⁹ using a mouse model with the human myocilin gene implicate the involvement of a peroxisomal targeting signal-1 receptor (PTS1R), which is absent from the mouse gene, in the disease process.

Molecular chaperones interact with partially folded or incorrectly folded polypeptides.^{50,51} They serve protective roles against various forms of cellular stress, including oxidative stress, heat shock, and chemical denaturation. They also assist in normal protein folding by adjusting the time frame of domain interactions or delaying folding until the correct compartment or organelle has been reached.⁵² Lens α -crystallins have been shown to act as molecular chaperones by protecting other crystallins and lens proteins against thermally induced aggregation,^{53,54} as well as oxidative stress.⁵⁵ Levels of α B-crystallin are increased within the TM as a consequence of heat shock, mechanical stress, and exposure to hydrogen peroxide.^{56–59} Two heat shock proteins, GroEL and Hsp90, also display chaperone activity by suppressing the aggregation of chemically denatured citrate synthase.^{60,61} Given that myocilin has been implicated as a stress response protein,^{10,62} studies were conducted to evaluate its potential to act as a general molecular chaperone by preventing the thermal inactivation of citrate synthase, *DrdI*, and GAPDH, as well as the thermally induced aggregation of citrate synthase. These proteins were chosen not because of their relevance to the TM, which is unknown, but, as general molecular chaperone targets for protection, because they have been studied in numerous other

From the ¹Casey Eye Institute, Oregon Health & Science University, Portland, Oregon; the ²Department of Chemistry, University of Portland, Portland, Oregon; and the ³Department of Pathology, University of Chicago Medical Center, Chicago, Illinois.

Supported by Research Corporation Cottrell College Science Award CC5860; M.J. Murdock Charitable Trust Grant 2006307; National Institutes of Health Grants EY003279, EY008247, and EY010572; and an unrestricted grant to Casey Eye Institute from Research to Prevent Blindness.

Submitted for publication April 11, 2011; revised July 7 and 28, 2011; accepted August 9, 2011.

Disclosure: A.M. Anderssohn, None; K. Cox, None; K. O'Malley, None; S. Dees, None; M. Hosseini, None; L. Boren, None; A. Wagner, None; J.M. Bradley, None; M.J. Kelley, None; T.S. Acott, None

Corresponding author: Ted S. Acott, Casey Eye Institute, Oregon Health & Science University, 3181 SW Sam Jackson Park Road, Portland, OR 97239; acott@ohsu.edu.

systems and are good choices for assessing chaperone effects.^{52–55,60,61} The molecular chaperone potential of myocilin was compared with that of bovine serum albumin, which has only poor chaperone activity.

MATERIALS AND METHODS

Cell Culture

Porcine TM cells were cultured in Dulbecco's modified Eagle's medium with 2.75 mg/mL glucose, 10% fetal calf serum, and 1% antibiotic/antimycotic mix containing 10,000 U/mL penicillin G, 10 mg/mL streptomycin sulfate, and 25 μ g/mL amphotericin B (Gibco/BRL, Carlsbad, CA) at 37°C in 100% humidity, 5% CO₂, and 95% air.⁶³ Confluent cultures were used by passage 5 and were serum-free for at least 24 hours before and during treatments.

Stress Induction of Myocilin

To produce mechanical stretch, porcine TM cells were grown to confluence for 3 days on 3.0- μ m pore cell culture insert membranes (Falcon; BD Biosciences, Franklin Lakes, NJ) in six-well plates and were made serum free. A glass bead was placed beneath the inserts and a weight was applied to the lids, forcing the insert down onto the bead and producing a mechanical stretch.⁶⁴ Cells were stretched for 24 hours before the collection of media. For cytokine treatments, porcine TM cells were maintained serum free for 48 hours before and during treatment for 48 hours with either 10 ng/mL recombinant human TNF α or 25 ng/mL recombinant human IL-1 α . For heat shock, confluent cells were made serum free for 48 hours before and during heat shock. To produce heat shock, cells were maintained at 44°C for 45 minutes and then returned to 37°C for 18 hours. Media were removed, and myocilin was concentrated using 30-kDa cutoff filters (Amicon; Millipore, Billerica, MA).

Purification, Verification, and Quantification of Myocilin

Myocilin was isolated from cultured media collected from porcine TM cells after treatment with 100 nM dexamethasone for 4 days. Media were immunoaffinity purified using affinity columns (Seize X Protein A-Sepharose; Pierce Biotechnology, Rockford, IL) to which a rabbit

polyclonal antibody had been attached. The antibody was produced against a peptide from the olfactomedin domain of myocilin to the sequence, SSYTSADATVNFAYDTGTGTSKIPFKNRC, by Triple Point Biologics (Forest Grove, OR). Although several other antibodies were compared with this one (data not shown), this antibody was used for all studies shown herein. An alternative purification method was used for some studies, including the thermal inactivation of GAPDH. For the citrate synthase studies, both types of purification were compared and produced similar results. Here, 1.5-mL aliquots of similar culture media were applied to diethylaminoethylamine (DEAE) anion exchange columns and eluted with 10 mM Bis-Tris buffer (pH 7.5) using NaCl gradients. Eluent fractions containing myocilin were further resolved on a TSK-gel DEAE-5PW column at room temperature using a high-performance liquid chromatography system (LC-20 AD; Shimadzu, Kyoto, Japan). Peaks were detected at 280 nm with a diode array detector (SPD-M20A; Shimadzu). Myocilin Western immunoblots were used to screen fractions. For further verification and identification of myocilin protein, liquid chromatographic-mass spectrometric (LC-MS/MS) amino acid sequencing was conducted using a capillary LC (1100 Series; Agilent, Santa Clara, CA) with electrospray ionization and an LTQ linear ion trap mass spectrometer (Thermo Fisher, San Jose, CA) by Larry David at the Department of Ophthalmology and the Oregon Health & Science University Proteomics Cores. The concentration of myocilin for chaperone experiments was determined using an extinction coefficient of 74,280 M⁻¹ cm⁻¹.

SDS-PAGE and Western Blot Analyses

Myocilin protein bands were analyzed using standard denaturing polyacrylamide gel electrophoresis techniques (SDS-PAGE) combined with either silver staining (Bio-Rad, Hercules, CA) or Western immunoblot analyses. Gels were run under strong reducing conditions with 100 mM dithiothreitol (DTT), using a 4.5% stacking gel and a 7.5% separating gel. The proteins were transferred to polyvinylidene difluoride or nitrocellulose membranes and were blocked with either 5% bovine serum albumin in TBST (10 mM Tris, pH 7.4, 150 mM NaCl, 0.01% Tween-20) or 2% skim milk in TBS (same buffer without Tween). Blots were washed extensively before and after incubation with the appropriate secondary antibodies conjugated to horseradish peroxidase. Subsequent blots were subjected to chemiluminescence detection (West Pico SuperSignal; Pierce) according to the manufacturer's in-

FIGURE 1. Stress induction of myocilin. Myocilin levels in culture medium were analyzed by Western immunoblots. Porcine TM cells were treated by (A) unstretched compared with mechanically stretched control for 24 hours. (B) Control (37°C) compared with heat shock (44°C) incubation for 45 minutes, both followed by incubation at 37°C for 24 hours. (C) Treatment with 10 ng/mL TNF α compared with vehicle control for 48 hours. (D) Treatment with 25 ng/mL IL-1 α compared with vehicle control for 48 hours. Data shown represent mean band densities of the 55-/57-kDa doublet, added together and plotted with SEM, where $n = 3$ to 5. The t -test significance is as indicated above each graph.

FIGURE 2. Immunoaffinity-purified myocilin from porcine TM cell medium. (A) Western immunoblots were probed with rabbit anti-human myocilin polyclonal antibody comparing 40 μ L of 10 $\times$ concentrated media (lane 1) and 10 μ L affinity-purified column eluent (lane 2). (B) Silver stain of SDS-PAGE of a similar affinity-purified column eluent. The positions of two molecular weight markers are shown on the left side of the gel. Arrows depict myocilin bands at approximately of 66 kDa and 55 to 57 kDa.

structions. Relative band densities of the 55-/57-kDa doublet were determined densitometrically (BioImage, Ann Arbor, MI; LabWorks, Upland, CA).

Thermal Inactivation of Citrate Synthase

The activity of porcine heart citrate synthase (Sigma, St. Louis, MO) was monitored in a manner similar to that described previously.⁶⁵ Citrate synthase catalyzes the formation of citrate in the presence of oxaloacetate and acetyl CoA, which was monitored at 412 nm using 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB) as a colorimetric reagent. The effects of thermal inactivation were investigated by diluting commercial stock citrate synthase (Sigma) with HEPES-KOH, pH 7.5, to a final concentration of either 1.5 μ M or 0.5 μ M in the presence or absence of BSA, myocilin, α -crystallin, HSP90, or RP1, the rabbit anti-myocilin antibody used in the immunoaffinity purification. Concentrations of chaperones or controls were selected based on the efficacy of effects and the literature. An aliquot of the enzyme solution was thermally inactivated at 55°C or 48°C for specific time periods. The controls were not heated or were heated in the absence of putative chaperones. At the end of the inactivation stage, 2 μ L enzyme solution was removed and added to 998 μ L reaction mixture containing 10 μ L of 10 mM oxaloacetate, 10 μ L of 10 mM DTNB, and 20 μ L of 7.5 mM acetyl CoA in TE buffer (50 mM Tris, pH 8.8, 2 mM EDTA). Reactions were carried out at room temperature, and absorbance at 412 nm was monitored over 20 minutes (Enzyme Kinetics software on a Cary 300 Bio UV-Visible Spectrophotometer; Varian Instruments, Palo Alto, CA).

Thermal Inactivation of GAPDH

The activity of rabbit muscle GAPDH (Sigma) was monitored in a manner similar to that previously described.⁶⁶ GAPDH catalyzes the oxidation of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate with the concomitant production of NADH, which was monitored spectrophotometrically at 340 nm. The enzymatic activity of GAPDH was assessed by diluting commercial stock GAPDH to a final concentration of 0.1 μ M in a reaction mixture containing 0.75 mM glyceraldehyde-3-phosphate, 2.73 mM DTT, 0.75 mM NAD⁺, and 0.03 M

sodium arsenate in 0.015 M sodium pyrophosphate buffer (pH 8.5) with or without chaperone. An aliquot of the enzyme solution was thermally inactivated at either 42°C or 45°C for 10 minutes and allowed to equilibrate to room temperature for 5 minutes before absorbance was monitored at 340 nm over a period of 20 minutes, as described. Controls were not heated or were heated in the absence of chaperones.

Thermal Aggregation and Light Scattering

A 100- μ L aliquot of stock 0.50 μ M citrate synthase solution in 40 mM HEPES-KOH, pH 7.5, was heat-inactivated in the presence or absence of either myocilin or BSA at 55°C for 5 minutes, returned to room temperature, and brought to a final concentration of 0.10 μ M in the same buffer. For light scattering measurements of citrate synthase thermal aggregation,^{53,61,67} the light source was an argon ion laser (162A-07; Spectra-Physics, Irvine, CA) using the 488-nm laser emission band with a 2-nm bandwidth cutoff and set at 7 mW power. Scattered light was measured at 90° to the light path and at 488 nm with a 2-nm bandwidth cutoff, using a spectrophotometer (USB2000; Ocean Optics, Dunedin, FL).^{53,61,67} Data were collected and analyzed using spectrometer operating software (OOIBase 32; Ocean Optics).

Restriction Digests

Reaction mixtures containing 1.2 μ L (6 U) *Dra*II, 3.0 μ L of 10 $\times$ NEB buffer 4, 0.1 ng myocilin or 2.0 μ g BSA, and water to total 28 μ L were incubated at 37°C for 5 minutes before heat inactivation at 58°C for various time periods. After the heat-inactivation period, 2 μ L (2.0 μ g) lambda DNA (New England Biolabs, Beverly, MA) was added to all

FIGURE 3. HPLC purification of myocilin from porcine TM cell medium. (A) HPLC elution profile showing protein absorbance at 280 nm (asterisk) indicating the position of the myocilin peak. Step elution gradient for NaCl is as shown. (B) Gels of material (asterisk) peak are shown after silver staining (lane 1) or immunostaining with myocilin antibody (lane 2). Lane 3 shows molecular weight markers with kilodalton migration, as indicated. Arrow indicates dominant myocilin immunostaining band at approximately 55 kDa.

samples. Digestions were carried out for 60 minutes at 37°C. At the end of the incubation period, 25 μ L of 2 $\times$ stop buffer (0.05% bromophenol blue and 50 mM disodium EDTA in 50% glycerol) was added, and 10 μ L samples were loaded onto 1% agarose gels containing 0.04% ethidium bromide. Gels were run at 100 V for 45 minutes before visualization using an ultraviolet light source. Relative band densities were quantified using bioimaging software (LabWorks; PerkinElmer, Waltham, MA).

Statistical Analysis

Statistical analysis for the determination of significance, when comparing control myocilin levels to stress responses levels in Figure 1, used Student's *t*-test. For heat effects in myocilin chaperone assays (Figs. 4–6), significance was determined using one-way ANOVA with Dunnett's multiple comparison posttest.

RESULTS

Stress Induction of Myocilin

Myocilin levels in porcine TM cell culture medium were significantly increased in response to several types of stress, including mechanical stretch, heat shock, and exposure to the cytokine IL-1 α or TNF- α (Fig. 1). The greatest relative response occurred after 24 hours for mechanical stretch or heat shock and after 48 hours of exposure to TNF α or IL-1 α . Figures show the mean relative band densities of the 55-/57-kDa doublet bands added together.

Partial Purification and Sequence Analysis of Myocilin

Western immunoblot analyses of immunoaffinity-purified secreted porcine myocilin yielded four major bands, including 1 at 66 kDa, a doublet around 55 kDa, and a light band at 50 kDa (Fig. 2A). Immunoaffinity purification increased yields approximately 12-fold (lane 2) compared with media that had been concentrated using concentrative filters (lane 1; Centricon; Millipore). Four major protein bands are also apparent on silver staining of similar gels (Fig. 2B).

An alternative purification of porcine myocilin was also achieved by anion exchange chromatography followed by HPLC separation. Stepwise elution from the HPLC column produced a significant immunoreactive fraction that eluted at 300 mM NaCl (Fig. 3A, asterisk). When this peak was subjected to SDS-PAGE followed by silver stain (lane 1) or Western immunoblot (lane 2) analysis, a primary myocilin band was seen at approximately 55 kDa (Fig. 3B). Additional minor bands were seen with both silver staining and immunoblots at approximately 66 kDa and 75 kDa.

When HPLC column eluents containing immunoreactive species were subjected to LC-MS/MS sequence analysis, eight unique myocilin peptides (IDTVGTDIR, KLFWKNFNMVTDYDIR, LFAWKNFNMVTDYDIR, LNPNLELER, NRYEYSSMIDYN-NPLEK, VHVLP, YELSTETLKAKE, and YEYSSMIDYNNPLEK) with nine unique spectra were identified. This represents approximately 14% sequence coverage of porcine myocilin.

Chaperone Effects on Thermal Inactivation of Citrate Synthase

The thermal inactivation of citrate synthase was reduced in a concentration-dependent manner by myocilin (Fig. 4A). At a molar ratio of 0.43 or 0.55 myocilin to citrate synthase, protection was better than that provided by BSA at a molar ratio of 40 BSA to citrate synthase (Fig. 4B). Even at 60 μ M, BSA was unable to provide protection equivalent to that of myocilin at 0.55 or 0.65 μ M. Optimal protection for 1.5 μ M citrate synthase was afforded by approximately 0.65 μ M myocilin; at a higher concentration of 0.82 μ M, myocilin actually inhibited enzyme activity. Nearly full retention of citrate synthase activity was observed after 5 minutes of thermal inactivation at 55°C, with slight differences noted in initial rates of reaction. Furthermore, approximately 0.65 μ M myocilin was capable of protecting citrate synthase activity after 10 minutes of heat inactivation with values equal to 50% that of control after 20 minutes (data not shown). Neither 1.5 μ M α -crystallin nor 0.043 mg/mL polyclonal rabbit anti-myocilin antibody used in purification was able to protect against the thermal inactivation of citrate synthase under identical experimental conditions

FIGURE 4. Protection against thermal inactivation of citrate synthase activity. Solutions containing 1.50 μ M citrate synthase and the indicated concentrations of either myocilin (A) or BSA (B) were heat inactivated at 55°C for 5 minutes before monitoring activity at 25°C for 20 minutes. One control (solid lines) was not heat inactivated, and another control (dashed line) was heat inactivated with no added myocilin or BSA. The data shown represent typical runs. (C) Solutions containing 1.5 μ M citrate synthase were not heated (1.5 μ M control) or were heated without additions (heat control) or with 0.65 μ M myocilin, 60 μ M BSA, 1.5 μ M α -crystallin, or 0.043 mg/mL RP1 (the anti-myocilin antibody used for immunoaffinity purification of myocilin). (D) Solutions containing 0.5 μ M citrate synthase were not heated (0.5 μ M control) or were heated without additions (heat control) or 2.0 μ M HSP90. Heat inactivation was at 48°C for 15 minutes before monitoring activity at 25°C for 20 minutes. (C, D) Mean values from experiments, where $n \geq 3$. * $P < 0.01$ compared with heat control, as determined by one-way ANOVA with Dunnett's multiple comparison test.

FIGURE 5. Protection of citrate synthase against heat-induced aggregation. Solutions containing 0.5 μ M citrate synthase were heated for 5 minutes at 55°C with no addition (control), 0.5 μ M BSA, 30 μ M BSA, or 0.22 μ M myocilin. Light scattering was then assessed using excitation and emission wavelengths of 488 nm. Means and standard errors are shown, where $n \geq 3$. * $P < 0.01$ compared with heated control using one-way ANOVA with Dunnett's multiple comparison test.

(Fig. 4C). HSP90 was able to provide significant protection of 0.5 μ M citrate synthase at 2.0 μ M (40% of controls), though still not as effectively as myocilin (Figs. 4C, 4D).

Light Scattering and Thermal Aggregation

Myocilin was also able to protect against the thermal aggregation of citrate synthase, as assessed by light scattering (Fig. 5). Solutions of 0.5 μ M citrate synthase that were not heated yielded values of approximately 30 relative scattering units (data not shown). When the same concentration of citrate synthase was heated for 5 minutes at 55°C and returned to room temperature and when scattering was monitored in an identical manner, values were increased >80-fold to approximately 2500 relative scattering units. BSA was able to partially protect against the aggregation of citrate synthase, but only at relatively high concentrations. A 1:1 coinubation of 0.50 μ M citrate synthase with BSA yielded values nearly identical to those of heated controls. However, when BSA was present in 60-fold excess over citrate synthase (30 μ M vs. 0.50 μ M, respectively), aggregation was significantly reduced, as noted by values that were approximately 60% of heat-inactivated controls. Myocilin was more efficient than BSA at suppressing thermal aggregation under identical experimental conditions. The presence of 0.22 μ M myocilin reduced light scattering of 0.5 μ M citrate synthase to values between 36% and 44% of heated controls.

Thermal Inactivation of GAPDH

GAPDH activity was significantly protected at 45°C in the presence of HSP90, with values equal to 62% of controls (Fig. 6A). Myocilin was able to protect GAPDH activity after 10 minutes of thermal inactivation at 45°C at levels similar to those afforded by α -crystallin after thermal inactivation at 42°C (values 26% vs. 29% of controls, respectively, Figs. 6A, 6B). α -Crystallin was not effective at 45°C (data not shown). The presence of 60 μ M BSA did not protect GAPDH activity under the experimental conditions examined.

Restriction Endonuclease Activity

Myocilin was also able to protect the activity of *DrdI* throughout the 30-minute time course of heat inactivation (Fig. 7). In the absence of myocilin, *DrdI* was fully inhibited after 30 minutes of heat inactivation at 58°C. Note that myocilin was more efficient than BSA at protecting *DrdI* activity, at 18 nM compared with 1 μ M, respectively, throughout the time course examined. The presence of both myocilin and BSA partially inhibited enzymatic activity in samples that were not heat treated (controls, 0 minutes).

DISCUSSION

The increased expression of myocilin by TM cells in response to stretch, heat shock, and the proinflammatory cytokines IL-1 α and TNF α suggests that it is a stress response protein. Previous studies^{8,10,13,17,62,68,69} showing increases in response to dexamethasone, hydrogen peroxide, TGF β , and mechanical stretch have been taken as evidence that it is a stress response protein in the TM. Recent mouse studies⁴⁹ suggest a similar stress component involving the PTS1R. Many of the myocilin mutants associated with open-angle glaucoma appear to trigger the unfolded or misfolded protein response, and neither the normal nor the mutant myocilin is secreted.^{10,43–48,70} This unfolded protein response^{42,71} would likely trigger increased expression of cellular stress proteins, perhaps including myocilin, which would exacerbate the problem and further interfere with normal TM cell function. Because the primary function of TM cells appears to be maintenance of aqueous humor outflow resistance and IOP homeostasis,⁷² impairment of overall TM cell function or reduction in TM cell numbers^{73–77} caused by a mutant myocilin-induced stress response could be important in the IOP elevations associated with glaucoma.

Stress response proteins often serve molecular chaperone functions.^{53,56,78–80} Although myocilin has been studied extensively for well over 10 years, identification of its normal function in the TM or other tissues has been elusive.^{10,81}

FIGURE 6. Protection of GAPDH activity against thermal inactivation. Solutions containing 0.1 μ M GAPDH were not heated (control) or were heated for 10 minutes at 45°C (A) or 42°C (B) with no addition (labeled 45°C 10 minutes or 42°C 10 minutes) or with the addition of 0.4 μ M HSP90, 0.2 μ M myocilin, 0.2 μ M α -crystallin, or 60 μ M BSA, as indicated. GAPDH enzyme activity is shown as means with standard errors for $n \geq 3$. * $P < 0.01$ compared with heated with no additional activities, as determined by one-way ANOVA with Dunnett's multiple comparison posttest.

Band	Control			18 nM myocilin			1 μ M BSA		
	0 min	20 min	30 min	0 min	20 min	30 min	0 min	20 min	30 min
5.1 kb	100%	44.0%	0.0%	101.7%	108%	71.7%	74.3%	134%	60.3%
4 kb	100%	0.0%	0.0%	72.8%	67.4%	12.9%	59.6%	34.8%	0.0%
2 kb	100%	0.0%	0.0%	37.6%	12.8%	0.0%	26.6%	0.0%	0.0%

FIGURE 7. Protection of *DrdI* restriction endonuclease activity from heat inactivation. The ability of *DrdI* to cleave 2 μ g λ DNA was assessed after heat inactivation for 0, 20, or 30 minutes, as indicated in the table above the lane. Control had no addition, and, as indicated, other lanes had 18 nM myocilin or 1 μ M BSA. A typical ethidium bromide-stained gel is shown with the uncleaved band at approximately 5.1 kb, the cleaved doublet at approximately 4 kb, and a single band at approximately 2 kb. Values in the table represent means of band fraction distributions determined from scans of gel lanes, where experiments were performed in triplicate.

Myocilin was shown here to be a relatively effective general molecular chaperone for three different enzymes. Its efficacy compared favorably with that of α -crystallin and HSP90, both well-established molecular chaperones.^{53–54,78,79,82} Differences in the efficacy of these three proteins with the various proteins that were chaperoned is not surprising because the chaperone process is complex and often molecule specific.^{83,84} Even at relatively high concentrations, BSA was a relatively poor chaperone.

One limitation of this study should be mentioned. Neither method for purification of myocilin resulted in absolutely homogeneous and pure protein, although in both preparations myocilin was the predominant protein. We compared the efficacy of the two preparations by a single chaperone assay and found them to be similar. In addition, the primary contaminants in the two preparations were either IgG that leached from the column or BSA. Neither was an effective chaperone when added at much higher concentrations than it was in the preparations. We also did not determine for certain which of the myocilin isoforms was responsible for or more effective as a chaperone. Myocilin isoforms are normally seen on gels at approximately 66 kDa, a doublet at approximately 55/57 kDa, and degradation products at 50 kDa, 37 kDa, and lower. Our two preparations exhibited some differences in isoform distribution, with both containing significant amounts of the 55-/57-kDa forms, but we could not definitively determine from these differences which isoform was active. Presumably, the 55-/57-kDa doublet is the most important, and some question about the 66 kDa form has been voiced.⁸⁴ Supporting, but certainly not proving, this point is the observation that we were unable to obtain a clear myocilin sequence from the 66-kDa band, whereas the 55-/57-kDa bands produced numerous myocilin peptides.

Molecular chaperones have been studied extensively in other systems.^{53,79,85,86} Thermal denaturation of proteins often exposes hydrophobic residues that are normally sequestered on the interior of the protein.⁸⁶ Formation of denatured protein aggregates is often driven by hydrophobic interactions. Molecular chaperones may provide transient alternative hydrophobic surfaces for these denaturing proteins in addition to assisting in the refolding process.^{83,85} Molecular chaperones often form large multimeric complexes of 2 to 24 or more subunits. These complexes are roughly doughnut-shaped structures that hold the chaperoned protein within the hydrophobic doughnut hole.^{86–88} The existence of various myocilin homomultimers has also been reported,^{36,43,89} supporting the possibility of such structures.

The mechanism by which myocilin protects these enzymes from thermal denaturation probably resembles that of other chaperones. If myocilin does serve as a molecular chaperone, it may do so at the cell surface or in the extracellular matrix. Extracellular chaperones have not been studied, but presumably they would resemble cellular chaperones. Myocilin has been considered a matricellular protein, although it may not fit the classic definition.^{30,90,91} Because matricellular proteins generally bind to several other proteins or cell surface receptors and modify their structural organization or facilitate changes in these interactions, such as occurs during cell division or ECM remodeling,⁹² there are some functional similarities. Evidence that myocilin actually serves a chaperone or matricellular protein function in the TM remains to be established, but these studies are compatible with a chaperone function for myocilin.

Acknowledgments

The authors thank Kevin Cantrell (University of Portland) for assistance with the light scattering apparatus and Genevieve Long for editorial assistance.

References

- Quigley HA. Number of people with glaucoma worldwide. *Br J Ophthalmol*. 1996;80:389–393.
- Shields MB. *Textbook of Glaucoma*. 4th ed. Baltimore: Williams & Wilkins; 1998.
- Stone EM, Fingert JH, Alward WLM, et al. Identification of a gene that causes primary open angle glaucoma. *Science*. 1997;275:668–670.
- Alward WL, Fingert JH, Coote MA, et al. Clinical features associated with mutations in the chromosome 1 open-angle glaucoma gene (GLC1A). *N Engl J Med*. 1998;338:1022–1027.
- Michels-Rautenstrauss KG, Mardin CY, Budde WM, et al. Juvenile open angle glaucoma: fine mapping of the TIGR gene to 1q24.3-q25.2 and mutation analysis. *Hum Genet*. 1998;102:103–106.
- Wiggs JL, Allingham RR, Vollrath D, et al. Prevalence of mutations in TIGR/myocilin in patients with adult and juvenile primary open-angle glaucoma. *Am J Hum Genet*. 1998;63:1549–1552.
- Polansky JR, Fauss DJ, Chen P, et al. Cellular pharmacology and molecular biology of the trabecular meshwork inducible glucocorticoid response gene product. *Ophthalmologica*. 1997;211:126–139.
- Nguyen T, Chen P, Huang W, Chen H, Johnson D, Polansky J. Gene structure and properties of TIGR, an olfactomedin-related glycoprotein cloned from glucocorticoid-induced trabecular meshwork cells. *J Biol Chem*. 1998;273:6341–6350.

9. Kubota R, Noda S, Wang Y, et al. A novel myosin-like protein (myocilin) expressed in the connecting cilium of the photoreceptor: molecular cloning, tissue expression, and chromosomal mapping. *Genomics*. 1997;41:360–369.
10. Tamm E. Myocilin and glaucoma: facts and ideas. *Retinal Eye Res*. 2002;21:395–428.
11. Anholt RR, Petro AE, Rivers AM. Identification of a group of novel membrane proteins unique to chemosensory cilia of olfactory receptor cells. *Biochemistry*. 1990;29:3366–3373.
12. Russell P, Tamm ER, Grehn FJ, Picht G, Johnson M. The presence and properties of myocilin in the aqueous humor. *Invest Ophthalmol Vis Sci*. 2001;42:983–986.
13. Lütjen-Drecoll E, May CA, Polansky JR, Johnson DH, Bloemendal H, Nguyen TD. Localization of the stress proteins alpha B-crystallin and trabecular meshwork inducible glucocorticoid response protein in normal and glaucomatous trabecular meshwork. *Invest Ophthalmol Vis Sci*. 1998;39:517–525.
14. Ueda J, Wentz-Hunter K, Yue BY. Distribution of myocilin and extracellular matrix components in the juxtacanalicular tissue of human eyes. *Invest Ophthalmol Vis Sci*. 2002;43:1068–1076.
15. Karali A, Russell P, Stefani FH, Tamm ER. Localization of myocilin/trabecular meshwork-inducible glucocorticoid response protein in the human eye. *Invest Ophthalmol Vis Sci*. 2000;41:729–740.
16. Swiderski RE, Ross JL, Fingert JH, et al. Localization of MYOC transcripts in human eye and optic nerve by in situ hybridization. *Invest Ophthalmol Vis Sci*. 2000;41:3420–3428.
17. Kulkarni NH, Karavanich CA, Atchley WR, Anholt RR. Characterization and differential expression of a human gene family of olfactomedin-related proteins. *Genet Res*. 2000;76:41–50.
18. Adam MF, Belmouden A, Binisti P, et al. Recurrent mutations in a single exon encoding the evolutionarily conserved olfactomedin-homology domain of TIGR in familial open-angle glaucoma. *Hum Mol Genet*. 1997;6:2091–2097.
19. Fingert JH, Ying L, Swiderski RE, et al. Characterization and comparison of the human and mouse GLC1A glaucoma genes. *Genome Res*. 1998;8:377–384.
20. Goldwirth A, Baulmann DC, Ohlmann A, Flugel-Koch C, Schocklmann H, Tamm ER. Myocilin is expressed in the glomerulus of the kidney and induced in mesangioproliferative glomerulonephritis. *Kidney Int*. 2005;67:140–151.
21. Ohlmann A, Goldwirth A, Flugel-Koch C, Fuchs AV, Schwager K, Tamm ER. Secreted glycoprotein myocilin is a component of the myelin sheath in peripheral nerves. *Glia*. 2003;43:128–140.
22. Ueda J, Wentz-Hunter KK, Cheng EL, Fukuchi T, Abe H, Yue BY. Ultrastructural localization of myocilin in human trabecular meshwork cells and tissues. *J Histochem Cytochem*. 2000;48:1321–1330.
23. Sakai H, Shen X, Koga T, et al. Mitochondrial association of myocilin, product of a glaucoma gene, in human trabecular meshwork cells. *J Cell Physiol*. 2007;213:775–784.
24. Wentz-Hunter K, Ueda J, Shimizu N, Yue BY. Myocilin is associated with mitochondria in human trabecular meshwork cells. *J Cell Physiol*. 2002;190:46–53.
25. Stamer WD, Perkumas KM, Hoffman EA, Roberts BC, Epstein DL, McKay BS. Coiled-coil targeting of myocilin to intracellular membranes. *Exp Eye Res*. 2006;83:1386–1395.
26. Hoffman EA, Perkumas KM, Highstrom LM, Stamer WD. Regulation of myocilin-associated exosome release from human trabecular meshwork cells. *Invest Ophthalmol Vis Sci*. 2009;50:1313–1318.
27. Goldwirth A, Scholz M, Tamm ER. Myocilin promotes substrate adhesion, spreading and formation of focal contacts in podocytes and mesangial cells. *Histochem Cell Biol*. 2009;131:167–180.
28. Ueda J, Yue BY. Distribution of myocilin and extracellular matrix components in the corneoscleral meshwork of human eyes. *Invest Ophthalmol Vis Sci*. 2003;44:4772–4779.
29. Filla MS, Liu X, Nguyen TD, et al. In vitro localization of TIGR/MYOC in trabecular meshwork extracellular matrix and binding to fibronectin. *Invest Ophthalmol Vis Sci*. 2002;43:151–161.
30. Wentz-Hunter K, Kubota R, Shen X, Yue BY. Extracellular myocilin affects activity of human trabecular meshwork cells. *J Cell Physiol*. 2004;200:45–52.
31. Wentz-Hunter K, Ueda J, Yue BY. Protein interactions with myocilin. *Invest Ophthalmol Vis Sci*. 2002;43:176–182.
32. Tan JC, Peters DM, Kaufman PL. Recent developments in understanding the pathophysiology of elevated intraocular pressure. *Curr Opin Ophthalmol*. 2006;17:168–174.
33. Park BC, Shen X, Fautsch MP, Tibudan M, Johnson DH, Yue BY. Optimized bacterial expression of myocilin proteins and functional comparison of bacterial and eukaryotic myocilins. *Mol Vis*. 2006;12:832–840.
34. Fautsch MP, Vrabel AM, Johnson DH. The identification of myocilin-associated proteins in the human trabecular meshwork. *Exp Eye Res*. 2006;82:1046–1052.
35. Torrado M, Trivedi R, Zinovieva R, Karavanova I, Tomarev SI. Optimedin: a novel olfactomedin-related protein that interacts with myocilin. *Hum Mol Genet*. 2002;11:1291–1301.
36. Gobeil S, Letarte L, Raymond V. Functional analysis of the glaucoma-causing TIGR/myocilin protein: integrity of amino-terminal coiled-coil regions and olfactomedin homology domain is essential for extracellular adhesion and secretion. *Exp Eye Res*. 2006;82:1017–1029.
37. Kim BS, Savinova OV, Reedy MV, et al. Targeted disruption of the myocilin gene (Myoc) suggests that human glaucoma-causing mutations are gain of function. *Mol Cell Biol*. 2001;21:7707–7713.
38. Gould DB, Miceli-Libby L, Savinova OV, et al. Genetically increasing Myoc expression supports a necessary pathologic role of abnormal proteins in glaucoma. *Mol Cell Biol*. 2004;24:9019–9025.
39. Zillig M, Wurm A, Grehn FJ, Russell P, Tamm ER. Overexpression and properties of wild-type and Tyr437His mutated myocilin in the eyes of transgenic mice. *Invest Ophthalmol Vis Sci*. 2005;46:223–234.
40. Gould DB, Reedy M, Wilson LA, Smith RS, Johnson RL, John SW. Mutant myocilin nonsecretion in vivo is not sufficient to cause glaucoma. *Mol Cell Biol*. 2006;26:8427–8436.
41. Fautsch MP, Bahler CK, Vrabel AM, et al. Perfusion of his-tagged eukaryotic myocilin increases outflow resistance in human anterior segments in the presence of aqueous humor. *Invest Ophthalmol Vis Sci*. 2006;47:213–221.
42. Zhang K, Kaufman RJ. The unfolded protein response: a stress signaling pathway critical for health and disease. *Neurology*. 2006;66:S102–S109.
43. Gobeil S, Rodrigue MA, Moisan S, et al. Intracellular sequestration of hetero-oligomers formed by wild-type and glaucoma-causing myocilin mutants. *Invest Ophthalmol Vis Sci*. 2004;45:3560–3567.
44. Caballero M, Rowlette LL, Borras T. Altered secretion of a TIGR/MYOC mutant lacking the olfactomedin domain. *Biochim Biophys Acta*. 2000;1502:447–460.
45. Yam GH, Gaplovska-Kysela K, Zuber C, Roth J. Aggregated myocilin induces Russell bodies and causes apoptosis: implications for the pathogenesis of myocilin-caused primary open-angle glaucoma. *Am J Pathol*. 2007;170:100–109.
46. Jacobson N, Andrews M, Shepard AR, et al. Non-secretion of mutant proteins of the glaucoma gene myocilin in cultured trabecular meshwork cells and in aqueous humor. *Hum Mol Genet*. 2001;10:117–125.
47. Zhou Z, Vollrath D. A cellular assay distinguishes normal and mutant TIGR/myocilin protein. *Hum Mol Genet*. 1999;8:2221–2228.
48. Zimmerman CC, Lingappa VR, Richards JE, Rozsa FW, Lichter PR, Polansky JR. A trabecular meshwork glucocorticoid response (TIGR) gene mutation affects translocational processing. *Mol Vis*. 1999;5:19.
49. Shepard AR, Jacobson N, Millar JC, et al. Glaucoma-causing myocilin mutants require the peroxisomal targeting signal-1 receptor (PTS1R) to elevate intraocular pressure. *Hum Mol Genet*. 2007;16:609–617.
50. Rothman JE. Polypeptide chain binding proteins: catalysts of protein folding and related processes in cells. *Cell*. 1989;59:591–601.
51. Pelham HR. Speculations on the functions of the major heat shock and glucose-regulated proteins. *Cell*. 1986;46:959–961.
52. Hartl FU. Molecular chaperones in cellular protein folding. *Nature*. 1996;381:571–579.
53. Horwitz J. α -Crystallin can function as a molecular chaperone. *Proc Natl Acad Sci U S A*. 1992;89:10449–10453.

54. Kelley M, David L, Iwasaki N, Wright J, Shearer T. α -Crystalline chaperone activity is reduced by calpain II in vitro and in selenite cataract. *J Biol Chem*. 1993;268:18844–18849.
55. Wang K, Spector A. The chaperone activity of bovine alpha crystallin: interaction with other lens crystallins in native and denatured states. *J Biol Chem*. 1994;269:13601–13608.
56. Tamm ER, Russell P, Johnson DH, Piatigorsky J. Human and monkey trabecular meshwork accumulate alpha B-crystallin in response to heat shock and oxidative stress. *Invest Ophthalmol Vis Sci*. 1996;37:2402–2413.
57. Fuchshofer R, Welge-Lüssen U, Lutjen-Drecoll E, Birke M. Biochemical and morphological analysis of basement membrane component expression in corneoscleral and cribriform human trabecular meshwork cells. *Invest Ophthalmol Vis Sci*. 2006;47:794–801.
58. Welge-Lüssen U, May CA, Eichhorn M, Bloemendal H, Lutjen-Drecoll E. α B-Crystallin in the trabecular meshwork is inducible by transforming growth factor-beta. *Invest Ophthalmol Vis Sci*. 1999;40:2235–2241.
59. Vittal V, Rose A, Gregory KE, Kelley MJ, Acott TS. Changes in gene expression by trabecular meshwork cells in response to mechanical stretching. *Invest Ophthalmol Vis Sci*. 2005;46:2857–2868.
60. Buchner J, Schmidt M, Fuchs M, et al. GroE facilitates refolding of citrate synthase by suppressing aggregation. *Biochemistry*. 1991;30:1586–1591.
61. Wiech H, Buchner J, Zimmermann R, Jakob U. Hsp90 chaperones protein folding in vitro. *Nature*. 1992;358:169–170.
62. Johnson D. Myocilin and glaucoma: a TIGR by the tail? *Arch Ophthalmol*. 2000;118:974–978.
63. Alexander JP, Acott TS. Involvement of the Erk-MAP kinase pathway in TNF- α regulation of trabecular metalloproteinases and TIMPs. *Invest Ophthalmol Vis Sci*. 2003;44:164–169.
64. Bradley JMB, Kelley MJ, Zhu XH, Anderssohn AM, Alexander JP, Acott TS. Effects of mechanical stretching on trabecular matrix metalloproteinases. *Invest Ophthalmol Vis Sci*. 2001;42:1505–1513.
65. Buchner J, Grallert H, Jakob U. Analysis of chaperone function using citrate synthase as nonnative substrate protein. *Methods Enzymol*. 1998;290:323–338.
66. Cori GT, Slein MW, Cori CF. Crystalline d-glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle. *J Biol Chem*. 1948;173:605–618.
67. Cai H, Wang CC, Tsou CL. Chaperone-like activity of protein disulfide isomerase in the refolding of a protein with no disulfide bonds. *J Biol Chem*. 1994;269:24550–24552.
68. Tamm ER, Russell P, Epstein DL, Johnson DH, Piatigorsky J. Modulation of myocilin: TIGR expression in human trabecular meshwork. *Invest Ophthalmol Vis Sci*. 1999;40:2577–2582.
69. Clark AF, Steely HT, Dickerson JE Jr, et al. Glucocorticoid induction of the glaucoma gene MYOC in human and monkey trabecular meshwork cells and tissues. *Invest Ophthalmol Vis Sci*. 2001;42:1769–1780.
70. Vollrath D, Liu Y. Temperature sensitive secretion of mutant myocilins. *Exp Eye Res*. 2006;82:1030–1036.
71. Imaizumi K, Tohyama M. The regulation of unfolded protein response by OASIS, a transmembrane bZIP transcription factor, in astrocytes (in Japanese). *Nippon Yakurigaku Zasshi*. 2004;124:383–390.
72. Acott TS, Kelley MJ. Extracellular matrix in the trabecular meshwork. *Exp Eye Res*. 2008;86:543–561.
73. Alvarado J, Murphy C, Juster R. Trabecular meshwork cellularity in primary open-angle glaucoma and nonglaucomatous normals. *Ophthalmology*. 1984;91:564–579.
74. Alvarado J, Murphy C, Polansky J, Juster R. Age-related changes in trabecular meshwork cellularity. *Invest Ophthalmol Vis Sci*. 1981;21:714–727.
75. Grierson I, Howes RC. Age-related depletion of the cell population in the human trabecular meshwork. *Eye*. 1987;1(pt 2):204–210.
76. Tschumper RC, Johnson DH. Trabecular meshwork cellularity: differences between fellow eyes. *Invest Ophthalmol Vis Sci*. 1990;31:1327–1331.
77. Liton PB, Challa P, Stinnett S, Luna C, Epstein DL, Gonzalez P. Cellular senescence in the glaucomatous outflow pathway. *Exp Gerontol*. 2005;40:745–748.
78. Ellis R. Chaperone function: the orthodox view. In: Henderson B, Pockley A, eds. *Molecular Chaperones and Cell Signaling*. New York: Cambridge University Press; 2006:3–21.
79. Horwitz J, Huang Q, Ding L, Bova M. Lens alpha-crystallin: chaperone-like properties. *Methods Enzymol*. 1998;290:365–383.
80. Sun Y, MacRae TH. Small heat shock proteins: molecular structure and chaperone function. *Cell Mol Life Sci*. 2005;62:2460–2476.
81. Tamm ER, Russell P. The role of myocilin/TIGR in glaucoma: results of the Glaucoma Research Foundation Catalyst Meeting in Berkeley, California, March 2000. *J Glaucoma*. 2001;10:329–339.
82. Bose S, Weikl T, Bugl H, Buchner J. Chaperone function of Hsp90-associated proteins. *Science*. 1996;274:1715–1717.
83. Das KP, Surewicz WK. On the substrate specificity of alpha-crystallin as a molecular chaperone. *Biochem J*. 1995;311(pt 2):367–370.
84. Ezzat M-K, Howell KG, Bahler CK, et al. Characterization of monoclonal antibodies against the glaucoma-associated protein myocilin. *Exp Eye Res*. 2008;87:376–384.
85. Das S, Mandal M, Chakraborti T, Mandal A, Chakraborti S. Structure and evolutionary aspects of matrix metalloproteinases: a brief overview. *Mol Cell Biochem*. 2003;253:31–40.
86. Das KP, Choo-Smith LP, Petrash JM, Surewicz WK. Insight into the secondary structure of non-native proteins bound to a molecular chaperone alpha-crystallin: an isotope-edited infrared spectroscopic study. *J Biol Chem*. 1999;274:33209–33212.
87. Das KP, Surewicz WK. Temperature-induced exposure of hydrophobic surfaces and its effect on the chaperone activity of alpha-crystallin. *FEBS Lett*. 1995;369:321–325.
88. Horwich A, Weber-Ban E, Finley D. Chaperone rings in protein folding and degradation. *Proc Natl Acad Sci U S A*. 1999;96:11033–11040.
89. Fautsch MP, Johnson DH. Characterization of myocilin-myocilin interactions. *Invest Ophthalmol Vis Sci*. 2001;42:2324–2331.
90. Wentz-Hunter K, Shen X, Okazaki K, Tanihara H, Yue BY. Overexpression of myocilin in cultured human trabecular meshwork cells. *Exp Cell Res*. 2004;297:39–48.
91. Gruber HE, Ingram JA, Hanley EN Jr. Cellular immunohistochemical localization of the extracellular protein myocilin in the intervertebral disc. *Biotechnol Histochem*. 2006;81:119–124.
92. Bornstein P, Sage EH. Extracellular matrix proteins: extracellular modulators of cell function. *Curr Opin Cell Biol*. 2002;14:608–616.