Perfusion with the Olfactomedin Domain of Myocilin Does Not Affect Outflow Facility

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PURPOSE. Mutations in the MYOC gene coding for myocilin are associated with elevated intraocular pressure (IOP), and recombinant myocilin, produced in a prokaryotic expression system, has been reported to affect aqueous outflow facility. This study was conducted to test whether perfusion with a fragment of recombinant myocilin (containing the full-length olfactomedin domain), produced in a eukaryotic expression system, affects facility.

METHODS. 293 EBNA cells were transfected by a vector containing the BM40 signal peptide, a human cDNA coding for myocilin, and a polyhistidine tag (HisTag) sequence. Recombinant protein was isolated by Ni-chelate chromatography, and characterized, and perfused into cultured anterior segments of human and porcine eyes.

RESULTS. Recombinant myocilin was secreted as a ~55-kDa intact protein and two fragments arising from cleavage of the recombinant protein at amino acid 215. The C-terminal fragment, containing the entire olfactomedin domain, was successfully isolated. When perfused into human and porcine eyes, this C-terminal fragment did not appreciably affect outflow facility.

CONCLUSIONS. Although the olfactomedin domain appears to be important for the function of myocilin, perfusion with a recombinant myocilin fragment containing this domain does not change outflow facility. It is possible that both the olfactomedin and N-terminal domains (including the leucine zipper) must be present for myocilin to have full function. Alternatively, posttranslational modifications of myocilin may have a major impact on protein function. (Invest Ophtalmol Vis Sci. 2003;44:1953–1961) DOI:10.1167/ iovs.02-08463

Glaucoma, an important cause of blindness,1 is characterized by a continuous loss of axons in the optic nerve head. Elevated intraocular pressure (IOP) is the major risk factor for axonal loss in glaucoma. Mutations in the gene coding for myocilin have been shown to be causative for autosomal-dominant, juvenile primary open-angle glaucoma (POAG), and some forms of adult-onset POAG.2–5 Patients with myocilin mutations may have higher IOP and a more severe course of disease than those with other forms of POAG.6–10 Despite significant research efforts, the function of myocilin in the normal and glaucomatous eye is poorly understood. Myocilin is a 55- to 57-kDa glycoprotein, containing an N-terminal leucine zipper region and a C-terminal olfactomedin domain.7,8 Most of the disease-causing mutations have been identified in the olfactomedin domain of myocilin. Myocilin can interact with itself to form high-molecular-mass aggregates, probably dimers and multimers.9,10 Myocilin–myocilin interactions occur mainly in the N-terminal region, and the leucine zipper domain and disulfide bond formation appear to be most critical for it.10 Secreted myocilin is found in the aqueous humor,10 and trabecular meshwork (TM) cells produce significant amounts of myocilin mRNA.11–15 One possible function of myocilin is that it could interact with the TM and increase the hydraulic resistance of this tissue. For example, myocilin isolated from the aqueous humor binds to polycarbonate microporous filters having pore sizes similar to those found in the outflow pathway tissues.15 Moreover, it was recently shown that perfusion of recombinant myocilin from a bacterial (prokaryotic) expression system significantly increases aqueous outflow resistance in perfused anterior segment organ cultures.16 However, because of important differences in posttranslational modification between eukaryotic and prokaryotic systems, it was not known whether myocilin produced by eukaryotic cells would cause similar changes in aqueous outflow resistance. Caballero et al.17 perfused human anterior segments with an adenovirus containing a truncated human myocilin coding sequence lacking the olfactomedin domain. This resulted in a net facility increase of 25%—that is, in a direction opposite to that seen by Fautsch et al.16 after perfusion with prokaryotically produced full-length myocilin.

Our goal in this work was to determine whether perfusion of recombinant myocilin, produced by a eukaryotic expression system, would affect aqueous outflow facility. To answer this question we expressed myocilin in eukaryotic cells and collected and purified the secreted recombinant protein. The recombinant myocilin was found to undergo specific proteolysis that created an N-terminal fragment (containing the leucine zipper region) and a C-terminal fragment (containing the olfactomedin domain). Perfusion of the C-terminal fragment did not affect outflow facility in anterior segment perfused organ cultures.

MATERIALS AND METHODS

Plasmid Construction

Human myocilin cDNA was obtained from human TM cell cultures treated for 5 days with 10−7 M dexamethasone, as previously described.13 It was then amplified by PCR, with the following primer pairs: 5′-TGCCATAGCGATGCTGAGCAGG-3′ and 5′-TGCCATAGCGCTAAGCTGAGCAG-3′. This sequence was designed so that the PCR product contained Xhol and XbaI restriction cleavage sites and a C-terminal sequence of six histidine residues (HisTag), in addition to the full coding region minus the signal peptide sequence. After digestion of the PCR product with

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these restriction enzymes, we used standard techniques to ligate the construct into the eukaryotic expression plasmid pD47. This plasmid (kindly provided by Ernst Pöschl, University of Erlangen-Nürnberg, Erlangen, Germany) was derived from plasmid pCEP4 (Invitrogen, Karlsruhe, Germany) by insertion of the BM40 (osteocentin) signaling peptide to facilitate secretion of recombinant proteins. The sequence of the resultant plasmid was verified by sequencing in both directions using the following primers: 5'−GAACGTGTAGTGGAGCAG−3', 5'GGGTTTGTCCAAACTCATC−3', 5'−G418, 250 fi culturing for 3 to 4 days in this medium with added hygromycin at a added antibiotics (penicillin, 100 U/mL; streptomycin, 100 µg/mL; and G418, 250 µg/mL; all Invitrogen). Transfected cells were selected by culturing for 3 to 4 days in this medium with added hygromycin at a final concentration of 300 µg/mL (Invitrogen). Long-term cell culture was then performed in membrane-based cell culture devices (CELLine CL-1000; Integra Biosciences, Fernwald, Germany). The long-term culture medium was DMEM with added antibiotics (as described, including G418 and hygromycin). This medium was supplemented with 10% FCS. The retentate was then ultracentrifuged (10,000 °C, 5% CO2, while the IOP was continuously monitored and recorded with a computer system. As a positive control, cultured anterior segments were perfused with latrunculin-B which caused a marked and reversible increase in outflow facility. Similar effects of latrunculin-B have been observed in monkey eyes in situ. After an adaptation period of at least 24 hours, medium in the left anterior segment was exchanged with solution containing recombinant protein (protein concentration 14 µg/mL), and then the anterior

**Western Blot Analysis**

For two-dimensional (2D) gel electrophoresis, samples were subjected to isoelectric focusing on immobilized pH gradient strips (IPG ReadyStrip; Bio-Rad) with pH ranging from 3 to 10 in 8 M urea, 10 mM dithiothreitol (DTT), 0.2% (wt/vol) Bio-Lytes Ampholytes (Bio-Rad) in 4% (vol/vol) 3-[4-cholamidopropyl]-dimethylammonio-2-hydroxy-1-propanesulfonate (CHAPS) buffer. Molecular weight separation was performed on a 6% to 15% (wt/vol) SDS-polyacrylamide gel, followed by silver staining of the gel by standard techniques or blot transfer to a PVDF membrane, as described earlier. Most gels were run under reducing conditions, although selected gels were run under nonreducing conditions. The membrane was blocked with 5% (wt/vol) BSA and incubated with an anti-myocilin (1:2000) or anti-HisTag (1:2000) antibody. The anti-myocilin antibody was raised in rabbits immunized with the peptide TRDTARAVPPGSREVST and has been characterized. The anti-HisTag antibody was supplied by ABR Affinity Bioreagents (Golden, CO). An alkaline phosphatase–conjugated anti-rabbit IgG (Promega, Mannheim, Germany) was used as secondary antibody at 1:10,000 dilution. The protein bands were detected with a commercial system (CDP-Star enhanced chemiluminescence on a Lumri-Imager, Roche Molecular Biochemicals).

**Filter Obstruction Tests**

Previous work has shown that myocilin binds preferentially to polycarbonate filters and that perfusion of aqueous humor through such filters causes filter blockage. Therefore, we perfused several protein-containing solutions through different types of filters at a constant flow rate of 100 µL/min, using a previously described approach. The perfused solutions were porcine aqueous humor collected from freshly enucleated eyes by paracentesis (protein concentration: 4 mg/mL), purified fragment of recombinant myocilin (protein concentration 17 µg/mL) and bovine serum albumin in Dulbecco’s phosphate-buffered saline (Invitrogen), at a concentration to match the porcine aqueous humor or the purified fragment of recombinant myocilin. Before filter perfusion, all solutions were centrifuged at 20,000g for 30 minutes at 4°C and prefiltered through a PVDF filter (Milllex-GP, Millipore) with 0.2-µm pore size. The filters used for testing were polycarbonate track-etched filters (PVDF-coated; Nucleopore Corp., Pleasanton, CA) with a pore size of 0.2 µm, and polycrylatesulfone filters (Sutor; Gelman Laboratories) with average pore size of 0.2 µm. All filters were supplied with, or were trimmed to have, a diameter of 15 mm.

**Organ Culture**

Porcine eyes were obtained from an abattoir within 2 hours of death and transported immediately to the laboratory. The culture technique was similar to that described elsewhere. Eyes were sectioned at the equator, and the lens, vitreous, iris, retina, and choroid were removed. The interior aspects of the ciliary processes, were carefully dissected. After extensive washes with DMEM, the anterior segments were placed in a modified culture dish and perfused with DMEM, containing 1.5 mg/mL glucose with 1% FCS and added antibiotics (penicillin, 100 U/mL; streptomycin, 0.1 mg/mL; and gentamicin, 17 µg/mL; all from Invitrogen) at a flow rate of 4.5 µL/min. The anterior eye segments were maintained in an incubator at 37°C, 5% CO2, while the IOP was continuously monitored and recorded with a computer system. As a positive control, cultured anterior segments were perfused with latrunculin-B which caused a marked and reversible increase in outflow facility. Similar effects of latrunculin-B have been observed in monkey eyes in situ.

After an adaptation period of at least 24 hours, medium in the left anterior segment was exchanged with solution containing recombinant protein (protein concentration 14 µg/mL), and then the anterior
segment was further perfused with this solution for at least 20 hours corresponding to a total delivered recombinant protein dose of at least 75 μg. The contralateral control eye received a similar exchange and perfusion with control solution. At the end of the perfusion, fluid in the anterior segment was exchanged with 4% paraformaldehyde, and then the eyes were further perfused with 4% paraformaldehyde at 4.5 μL/min for at least 10 hours. After the eyes were dissected from the culture dishes, wedges containing outflow tissues were dissected and immersed in Ito’s fixative.29

Ostensibly normal human eyes were obtained from the Canada Eye Bank (Ontario Division) and prepared for organ culture using standard methods essentially as described earlier.23 The protocol adhered to the tenets of the Declaration of Helsinki for research involving human tissue. They were perfused at 2.5 μL/min with DMEM plus added antibiotics and 1% FCS for 24 to 48 hours for equilibration. Myocilin-containing and control solutions (shipped on dry ice from Germany to Toronto) were then administered as described earlier. Immediately before perfusion, solutions were centrifuged for 20 minutes at 3000 g at 4°C, and protein concentration in the supernatant was measured with the Bradford assay (Bio-Rad) to be in the range of 10 to 14 μg/mL. BSA (350 μg/mL) was then added to both control solution and myocilin-containing fluid. Perfusion with myocilin-containing fluid was continued for 16 to 20 hours, after which the perfusion syringes were refilled, and the perfusion was continued for a total perfusion time of up to 44 hours, corresponding to a total delivered recombinant protein dose of 66 to 92 μg. Eyes were then fixed as described earlier.

**Histology**

Tissue wedges fixed in Ito’s solution were dehydrated and embedded in Epon with standard techniques. Semithin sections were cut and stained with toluidine blue and analyzed by light microscopy. Eyes that showed substantial pigment accumulation within the meshwork, appreciable loss of TM cells, or other disruption of outflow tissue structures were discarded and not used in the calculations of facility change. Outflow tissues fixed in 4% paraformaldehyde were trimmed and either embedded in paraffin and sectioned using standard techniques or frozen in liquid nitrogen and used to cut cryosections. Sections were then incubated overnight at 4°C with 1:25 to 1:50 anti-HisTag antibodies, washed with PBS, and incubated for 1 hour at room temperature with secondary antibodies (1:2000) labeled with a fluorescent conjugate (Alexa Fluor; Molecular Probes Inc., Eugene, OR). Unfortunately, significant nonspecific labeling was observed that precluded reliable use of immunolabeled sections.

**RESULTS**

**Protein Isolation and Characterization**

Western blot analysis demonstrated that recombinant myocilin with a molecular mass of 55 to 57 kDa was expressed and secreted by the 293 EBNA cells. However, a significant amount of the recombinant myocilin was proteolyzed, yielding two major fragments. Figure 1 shows that one fragment migrated at 20 to 22 kDa and was recognized by two different antibodies directed against epitopes on the N-terminal end of the protein, whereas the second fragment migrated at 30 to 33 kDa and was recognized by antibodies against the C-terminal HisTag. One antibody recognized an additional band at 24 to 26 kDa (Fig. 1A). No bands were observed in supernatant from nontransfected control cells (not shown). Gels run under nonreducing conditions (Fig. 1E) showed that the recombinant protein formed multimers of various sizes that were not present under reducing conditions. Multimers were not recognized by the anti-HisTag antibody, possibly due to masking of the HisTag in multimers.

Supernatant from cells was purified by chromatography on a Ni-chelate column (Fig. 2A), after which collected fractions were subjected to one-dimensional (1-D) SDS-PAGE and either Western blot analysis or gel silver staining. Both Western blot analysis (not shown) and silver staining (Fig. 2A) showed that the recombinant protein was concentrated within a subset of the collected fractions. It was these purified fractions (Fig. 2A) that were pooled and used for dialysis and subsequent organ culture experiments.

In one set of experiments, we further characterized these fractions by first applying them to a chromatography column (Blue Sepharose; Amersham Pharmacia Biotech; Fig. 2B), followed by 1-D SDS-PAGE and either gel silver staining (Fig. 2B,C) or electroblot transfer to a PVDF membrane. After the membrane was stained with Coomassie blue dye, two bands of approximate molecular masses of 30 and 33 kDa, corresponding to putative recombinant myocilin fragments, were individually excised and characterized by Edman N-terminal sequencing. The N-terminal sequences of both fragments were LKSELTEV, corresponding to position amino acids 215-222 of myocilin. This suggests that these 30- to 33-kDa bands represented cleavage products of the recombinant protein. The different molecular masses of both excised bands are consistent with translational and posttranslational processing of recombinant myocilin.

To more completely characterize the recombinant proteins and their extent of purification, recombinant protein solutions were analyzed by 2-D SDS-PAGE, followed by either Western blot analysis or silver gel staining. Recombinant myocilin showed the same molecular mass and isoelectric point (pl) as myocilin from fresh human TM (Figs. 3A, 3B). The 2-D gels also revealed that the 22-kDa N-terminal fragment consisted of three major spots with pls between 6.6 and 8 (Fig. 3A). Based on our knowledge of the cleavage site of the N-terminal fragment, the pl of this fragment was calculated to be 7.2, which is consistent with the 2-D gels. After purification of the C-terminal myocilin fragment described earlier, one distinct spot (pl of approximately 5.2 and mass ~29 kDa) and one faint spot were labeled in silver-stained gels (Fig. 3C). These spots also stained with antibodies against the HisTag (Fig. 3D). Staining of the purified protein with N-terminal myocilin antibodies did not label OD-Myc, but visualized a spot with the same pl and mass as fresh myocilin, indicating that minor amounts of intact myocilin were copurified (data not shown).

**FIGURE 1.** 1-D SDS-PAGE Western blots of recombinant myocilin, showing both intact protein (~55 kDa) and fragments (~20 kDa and ~33 kDa). Recombinant protein was obtained from the supernatant of two batches of transfected 293 EBNA cell lines (lanes 1 and 2). Various antibodies were used: (A, E) an antibody directed against a peptide corresponding to amino acids (aa) 188-204 of myocilin; (B) an antibody directed against a fusion protein corresponding to aa 1-148 of myocilin; (C, D) an antibody directed against the HisTag. Under nonreducing conditions (D, E) the recombinant protein formed multimers that were not present under reducing conditions (A–C). Multimers were not recognized by the anti-HisTag antibody (D).
FIGURE 2. Characterization of recombinant myocilin during isolation and purification steps shows that the recombinant protein was cleaved at aa 215 (A). After Ni-chelate chromatography, myocilin-containing fractions were identified by SDS-PAGE and Western blot analysis (not shown); the corresponding fractions are underlined in the silver-stained gel. These fractions were pooled (Pool Post Chelate) and used for perfusion experiments, or further purified by chromatography (Blue Sepharose, Amersham) (B). After blue sepharose chromatography, myocilin-containing fractions were identified by SDS-PAGE and Western blot analysis (not shown), with corresponding fractions once again underlined in the silver-stained gel. These fractions were pooled (Pool Post Blue Sepharose), again analyzed by SDS-PAGE (C) and blotted. Putative myocilin bands were individually cut from the membrane and sequenced by Edman degradation, yielding the sequence shown in (C). M, molecular weight standards; S1, supernatant from cells before purification; T1, flow-through from Ni-chelate column; S2, pooled myocilin-containing fractions from Ni-chelate column; T2, flow-through from chromatography (Blue Sepharose) column; F, final pooled myocilin-containing fractions from Blue Sepharose column; used for sequencing.

FIGURE 3. 2-D SDS-PAGE followed by either Western SDS-PAGE or silver gel staining shows that the intact recombinant myocilin (A, arrowhead) had approximately the same molecular mass and pl as myocilin from fresh human TM (B, arrowhead). The 2-D gels also revealed that the 22-kDa N-terminal fragment seen on 1-D gels consisted of three major spots with pl values between 6.6 to 8 (A). After purification of the C-terminal myocilin fragment, a dominant spot (pl ~5.2) and a weak second spot were labeled in silver-stained gels (C, arrows). These spots were also labeled on Western blots with antibodies against the HisTag (D, arrows).
Perfusion Assays with Recombinant Protein

Both porcine aqueous humor and a solution of recombinant protein blocked polycarbonate filters in an in vitro assay. Perfusion of track-etched filters at a constant flow rate of 100 μL/min was begun at the time labeled Start, and the pump was turned off at the time labeled Stop. The control BSA solutions had protein concentrations matched to their respective experimental solutions (A, porcine aqueous humor: 4 mg/mL; B, recombinant protein: 17 μg/mL).

Perfusion of recombinant protein into cultured porcine anterior segments (n = 15 pairs with well-preserved cells, essentially normal TM histology and good facility traces) showed no significant effects on facility (Figs. 5A, 5B). The average starting facility for the porcine eyes was 0.50 ± 0.20 μL/min · mm Hg for control eyes and 0.49 ± 0.17 μL/min · mm Hg for experimental eyes (mean ± SD). The change in facility due to perfusion with control fluid was ±2.2% ± 30.4%, and the change in facility due to perfusion with recombinant protein was −3.3% ± 30.3%. The net (paired) facility change was −5.4% ± 27.9%, which was not significantly different from zero. Histologically, we observed no difference in outflow tissue structure between control and experimentally perfused eyes (Fig. 5C).

Similar results were obtained with cultured human anterior segments (n = 5 pairs) with good TM histology and facility traces (Figs. 6A, 6B). The average starting facility for the human eyes was 0.37 ± 0.36 μL/min · mm Hg for control eyes and...
0.26 ± 0.16 μL/min · mm Hg for experimental eyes (mean ± SD). The change in facility due to perfusion with control fluid after 19 hours of perfusion was −1.0% ± 18.6%, and the change in facility due to perfusion with recombinant protein was −6.4% ± 10.6%. The net (paired) facility change was −5.4% ± 25.4%, which was not significantly different from zero and was identical with the mean net change in porcine eyes. After 40 hours of perfusion with recombinant protein the results were similar, with a net facility change of −6.6% ± 21%, which again was not significantly different from zero.

To better characterize the interactions of recombinant protein with outflow tissues, we performed SDS-PAGE and Western blot analysis on samples perfused into porcine eyes and collected from the perfusion dishes at various times during the experiment. Figure 7 shows that there was a significant amount of the C-terminal fragment of the recombinant protein that entered the eye and passed through the TM, collecting in the perfused fluid outside the eye. Thus, although some recombinant protein may have interacted with the TM, a significant fraction appeared to pass freely through the outflow tissues. Furthermore, the fraction passing through the meshwork had a molecular mass similar to the protein perfused into the eye, suggesting that the recombinant protein was not modified by TM cells.

DISCUSSION

In this work, administration of a recombinant myocilin fragment containing the olfactomedin domain (Fig. B), prepared in a eukaryotic system, had little effect on outflow facility. The olfactomedin domain is highly conserved across mammalian
Further, most clinically significant mutations occur in this region and apparently inhibit extracellular secretion of the mutated protein. Finally, studies of myocilin interactions with optiomedin, an olfactomedin-like protein, show that these interactions seem to involve the olfactomedin domain. These facts together suggest that the olfactomedin domain of myocilin is very important for the function of this protein. It is therefore particularly interesting that perfusion of anterior segments with a recombinant protein fragment containing the olfactomedin domain yielded no appreciable change in outflow facility.

There are several possible interpretations of this result. One interpretation is that extracellular myocilin plays no direct role in influencing outflow facility and that the primary role of myocilin is intracellular. Some evidence exists to support this role: Although myocilin has a secretion signaling sequence and has been found in aqueous humor, it has also been reported to be associated with the cytoskeletal elements and mitochondria. A second interpretation is that extracellular myocilin plays a role in influencing outflow facility. In this case, our data show that this role cannot be directly mediated by the olfactomedin domain alone, but instead must also have the N-terminal region of the protein. This region of the protein includes the leucine zipper domain, which has been shown to be important in dimer and multimer formation. Multimer formation would be expected to be efficient at influencing aqueous outflow dynamics, particularly if it were to occur within the narrow flow regions of the juxtacanalicular tissue. In addition, the N-terminal region of the protein may contain binding sequences for extracellular matrix proteins, and the interaction of myocilin with extracellular proteins could be important in determining outflow facility. For example, myocilin is known to bind to the HepII domain of fibronectin, although a specific binding sequence on myocilin has not yet been identified.

In interpreting our results, it is important to keep in mind potential limitations of the organ culture system that we used to test the physiological effects of the recombinant protein.
this system, perfusions last for hours to days, rather than the years required for development of POAG. However, it is clear that our recombinant myocilin fragment was rapidly able to plug certain types of polymeric filters with pore sizes comparable to those found in the juxtacanalicular tissue, consistent with previous reports of myocilin fragment binding to such membranes.\(^{15}\) Considering the low protein concentration in the recombinant myocilin-fragment-containing solutions compared with the aqueous humor samples (17 \(\mu\)g/mL vs. 4 mg/mL) it appears that our purified recombinant protein fragment was very effective at blocking polymeric filters. The inability of this recombinant myocilin fragment to ‘plug’ the TM probably indicates the more complex physicochemical and biochemical environment present within TM.

Our results differ from those of Fautsch et al.,\(^{16}\) who observed significant reductions in facility due to perfusion with recombinant myocilin in human anterior segments. They used a bacterial expression system to produce recombinant myocilin, obtaining primarily full-length recombinant protein. The use of a bacterial expression system implies that normal eukaryotic posttranslational modifications were not performed, so that the recombinant myocilin in these earlier experiments could have differed significantly from our recombinant protein. Thus, the disparate facility outcomes could be due to differences arising from posttranslational modifications of the recombinant myocilin, to the need for full-length myocilin, or both. At the present time we do not have sufficient data to distinguish between these possibilities. In parallel experiments, we expressed mutant myocilin (Y437H) linked to the BM40 (osteonectin) signaling peptide in our expression system. We observed that the mutant myocilin was not secreted, corroborating findings by Jacobson et al.\(^{20}\)

It is of interest to better understand the events leading to cleavage of the recombinant myocilin. Unfortunately, we do not fully understand this process. In separate experiments with recombinant rat myocilin in the same expression system, cleavage occurred at the same site. This is consistent with the fact that the sequence containing the cleavage site, FQE-LKSELTEVPA, is highly conserved across several species (human, monkey, mouse, rat, bovine and porcine). Furthermore, it seems likely that this cleavage was not due to degradation of the protein, because the resultant fragments showed distinct molecular weights rather than a broad range of fragment sizes. Minor variations in the molecular weights and the pI values of the resultant fractions are likely to be due to posttranslational modification of the recombinant protein. A similar cleaved fragment was not observed in Western blot analysis of intact human TM (Fig. 5B). In aqueous humor samples, however, bands that were immunoreactive for myocilin antibodies and had lower molecular masses at approximately 15, 25, and 32 kDa were reported.\(^{15}\) These results indicate that fragments of myocilin are also present in the aqueous humor, although it is not clear whether the site of cleavage in vivo is similar to that in our experimental system. It is also far from clear whether the cleavage event could be induced under certain pathologic circumstances. We tried to prevent or inhibit this cleavage by adding a number of different commercially available broad-spectrum protease inhibitors to the cell culture media. Unfortunately, these efforts were unsuccessful.

**FIGURE 7.** SDS-PAGE confirms that recombinant protein was introduced into perfused porcine eyes and that appreciable amounts of recombinant protein passed through the outflow tissues during perfusions, apparently without modification. (A) A Western blot using antibody against HisTag; (B) a Coomassie blue-stained membrane. Lane 1: Cell supernatant before chromatography (positive control); lane 2: 0.2 \(\mu\)g recombinant protein after Ni-chelate chromatography and dialysis against DMEM; lane 3: DMEM dialysate (from outside dialysis tube) after dialysis; lane 4: DMEM with added recombinant protein (0.16 \(\mu\)g loaded in well) after dialysis and centrifugation (4000g for 20 minutes); lane 5: pellet of partially precipitated recombinant protein after centrifugation; lane 6: perfusion culture perfusate collected after passage through the eye during baseline perfusion without recombinant protein; lane 7: recombinant protein solution as delivered to the eye (14 \(\mu\)g/mL); lane 8: perfusion culture perfusate collected after passage through a control eye; and lane 9: perfusion culture perfusate collected after passage through a treated (experimental) eye.

**FIGURE 8.** The recombinant myocilin and the truncated form of the secreted protein cleaved at aa 215, as used in organ-culture perfusions. The anti-Myoc antibody epitope location is marked at the top.
In separate experiments, using transfected mouse MUMTNEI/1 TM cells, we obtained the same degradation product, but not the intact protein. This indicates that the degradation process is not specific to the EBNA 293 cells. Therefore, it is an open question as to how (or whether) it is possible to obtain full-length recombinant myocilin using a eukaryotic expression system. A significant amount of additional experimental work is necessary to answer this question.

In our hands, the full-length recombinant myocilin and the C-terminal fragments were quite susceptible to precipitation (Fig. 7, lanes 4 and 5). This may be related to the tendency of myocilin to form multimers, as we observed in our nonreducing SDS-PAGE results. We tried to prevent such precipitation by adding many different agents to the recombinant protein solutions. Of these, 5 mM calcium was the most effective, although even it did not completely prevent the precipitation. How precipitation is avoided in the aqueous humor and its physiological importance is unclear.

Acknowledgments

The authors thank Olaf Wendler and Klaus von der Mark (University of Erlangen-Nürnberg, Germany) for valuable discussions; the Canadian Eye Bank (Ontario Division) for kindly supplying human eyes; Antonia Kellenberger, Kathrin Baier, and Karin Gohler for expert technical assistance; and Terete Borràs (Duke University, Durham, NC) for providing antibodies against recombinant myocilin.

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