Functional Role of Proteolytic Processing of Recombinant Myocilin in Self-Aggregation

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PURPOSE. Recombinant myocilin expressed in cells in culture is endoproteolytically cleaved in the endoplasmic reticulum by calpain II, releasing an N-terminal and a C-terminal fragment. This proteolytic processing has been speculated to regulate the molecular interactions of myocilin. The main purpose of this study was to analyze the effect of the proteolytic cleavage on myocilin aggregation.

METHODS. cDNAs encoding human myocilin and the N- and C-terminal fragments were transiently expressed in HEK-293T cells. Covalent interactions of recombinant myocilin were analyzed by SDS-PAGE and Western immunoblot analysis in different dissociating conditions. Noncovalent interactions were studied by solid-phase binding assays, performed with Ni-chelating HPLC-purified recombinant proteins, and by Far-Western blot analysis.

RESULTS. Western blot analysis of recombinant myocilin aggregates under either increasing ionic strength or increasing concentration of reducing agent indicated that ionic interactions do not contribute to the stability of the molecular complexes linked by disulfide bridges. Disulfide myocilin homoaggregates decreased as the proteolytic processing increased. Solid-phase binding assays showed the existence of high-affinity ($K_d = 0.068 \mu\text{M}$) noncovalent myocilin-myocilin interactions and that processed fragments bound to the full-length protein with significantly reduced affinity. Far-Western blot analysis confirmed noncovalent interactions between recombinant myocilin disulfide aggregates.

CONCLUSIONS. The proteolytic processing of recombinant myocilin decreases myocilin homoaggregates. These data provide the first evidence of a functional role for this processing in myocilin aggregation and suggest that disulfide complexes of myocilin could organize into a dynamic extracellular network sustained by noncovalent N-terminal interactions. (*Invest Ophthalmol Vis Sci.* 2010;51:72-78) DOI:10.1167/iovs.09-4118

Myocilin is an extracellular glycoprotein present in muscular and ocular tissues such as iris, ciliary body (CB), and trabecular meshwork (TM).¹⁻⁶ It forms large molecular aggregates in the aqueous humor (AH).⁷⁻⁹ Mutations in the myocilin (*MYOC*) gene cause autosomal dominant juvenile glaucoma, and they are also present in a reduced proportion (3%-5%) of adult-onset primary open-angle glaucoma (POAG) in different populations.¹⁰⁻¹² POAG is a heterogeneous disease originated by the progressive apoptosis of the optic nerve,¹³ which constitutes a leading cause of blindness in developed countries. Myocilin presents a modular design composed of the N-terminal region (amino acids 1-202), the central linker domain (amino acids 202-244),^{14,15} and the compact¹⁶ C-terminal olfactomedin-like domain (amino acids 244-504).³ The N-terminal domain contains two coiled-coil (CC) domains¹⁷ and a leucine zipper-like motif³ in the second CC. These three modules are encoded by exons 1, 2, and 3 and coincide approximately with three independent folding domains.¹⁷ We have reported that recombinant myocilin undergoes an intracellular endoproteolytic cleavage by calpain II in the central linker domain of the polypeptide chain.¹⁵ The processing takes place in the endoplasmic reticulum and releases two fragments that contain the N- and C-terminal structural domains. The C-terminal fragment has been detected in both human and bovine CB and the AH, indicating that the proteolytic processing also occurs in ocular tissues.¹⁵ Of note, photomedin-1 and gliomedin, two olfactomedin-like domain-containing proteins, are cleaved in a similar fashion.^{18,19} Pathogenic mutations located in the olfactomedin-like domain reduce the specific cleavage of the recombinant protein.¹⁵

Despite many efforts having been made since the discovery of *MYOC* as a glaucoma gene in 1997,¹⁰ the function of this protein in normal and glaucomatous eyes remains poorly understood. Similarly, the functional meaning of the proteolytic processing of myocilin is currently unknown, although it has been suggested to contribute to the modulation of myocilin interactions.¹⁵

In the present study, the specific proteolytic cleavage of recombinant myocilin reduced its extracellular covalent aggregates. In addition, the results revealed the existence of noncovalent interactions between myocilin aggregates, which may play an important role in the extracellular function of the protein.

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Presented at the annual meeting of the Association for Research in Vision and Ophthalmology, Fort Lauderdale, Florida, May 2009.

Supported in part by research grants from the Spanish Ministry of Science and Innovation, the Regional Ministry of Health and the Regional Ministry of Science and Technology of the Board of the Communities of "Castilla-La Mancha," and "Instituto de Salud Carlos III" (SAF2008-02228; GCS-2006_C/12; PAI-05-002 and PCI08-0036; and RD07/0062/0014) (JE); National Institute of Health Grants EY04873 and EY00785 for core facilities (MC-P); and grants from Research to Prevent Blindness and The Connecticut Lions Foundation (MC-P). FM-R is the recipient of a fellowship from the Regional Ministry of Education and Science of the Board of the Communities of "Castilla-La Mancha."

Submitted for publication June 10, 2009; revised July 28, 2009; accepted August 7, 2009.

Disclosure: J.-D. Aroca-Aguilar, None; F. Martínez-Redondo, None; F. Sánchez-Sánchez, None; M. Coca-Prados, None; and J. Escribano, None

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FIGURE 1. Myocilin cDNA constructs used in the study. Boxes placed in the C-terminal ends represent myc (m), HA epitopes, and the His-tag (His), used to detect and purify the recombinant proteins. Numbers correspond to the amino acid location of the different myocilin regions. LD, linker domain; LZ, leucine zipper; OLF, olfactomedin domain; SP, myocilin signal peptide.

MATERIALS AND METHODS

cDNA Constructs and Expression of Recombinant Proteins

cDNA constructs encoding myocilin, its N- and C-terminal fragments, tagged with the myc epitope at their C-terminal ends and a cDNA encoding myocilin fused to the HA epitope at its C terminus (Fig. 1) were cloned in the pcDNA3.1 expression vector as previously reported.^{15,20,21} In addition, a cDNA encoding myocilin fused to the HA and myc epitopes at their N- and C-terminal ends,²¹ respectively, was used to analyze the fate of the two processed fragments (Fig. 1). All the recombinant proteins were fused to a 6XHis tail at their most C-terminal ends (Fig. 1) and were transiently expressed in human embryonic kidney 293T (HEK-293T) cells bought from the American Type Culture Collection (ATCC Manassas, VA), as previously described.^{15,21} Recombinant human myocilin used as a control for Western blot was expressed in HEK-293T cells using Opti-MEM (Invitrogen-Gibco, Carlsbad, CA) without fetal bovine serum.

Bovine Ocular Tissues

Bovine eyes were obtained from a local abattoir and dissected from the posterior pole by removing both the vitreous and the lens. After microdissecting the CB and the iris, we obtained the trabecular meshwork by making parallel cuts anterior to the scleral spur and posterior to Schwalbe's line. Tissues were homogenized as previously described.¹⁵

Polyacrylamide Gel Electrophoresis and Western Blot Analysis

Analytical polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) was performed using a gel electrophoresis system (Mini-Protein III; Bio-Rad, Hercules, CA). For reducing Western blot analysis, samples were incubated with loading buffer containing 100 mM β -mercaptoethanol at 95°C for 5 minutes. For nonreducing SDS-PAGE, samples were treated with loading buffer without β -mercaptoethanol at room temperature. After electrophoresis, the gels were transferred onto nitrocellulose membranes (Hybond ECL; Amersham, Uppsala, Sweden). The recombinant proteins were immunodetected with either mouse monoclonal anti-myc or with anti-HA (Santa Cruz Biotechnology, Santa Cruz, CA) as primary antibodies, diluted at 1:500. A horseradish peroxidase-conjugated antibody against mouse IgG (Pierce, Rockford, IL) was diluted at 1:1000. Chemiluminescence was then performed (Supersignal Dura Western Blot reagents; Pierce).

Purification of Recombinant Proteins

The different versions of recombinant myocilin were directly purified from conditioned culture medium by nickel-chelating high performance liquid chromatography (HPLC; a Hi-Trap Chelating HP 1-mL column, coupled to an Akta-Purifier chromatographer; Amersham Biosciences). Before HPLC fractionation, 20 mL of 5 $\times$ binding buffer (100

mM sodium phosphate [pH 7.4], 2.5 M NaCl, 37.5 mM imidazole) were added to 80 mL of culture medium containing each recombinant protein. Samples were loaded into the HPLC column with a peristaltic pump (Ismatec, Glattbrugg, Switzerland) at a flow rate of 1 mL/min. The column was eluted with 10 mL of binding buffer followed by a linear gradient of imidazole from 7.5 to 200 mM in the same buffer, over 14 minutes, at a flow rate of 0.7 mL/min. Fractions were collected and analyzed by Western blot analysis with an anti-myc antibody, to detect the recombinant proteins. The fractions containing the isolated proteins were pooled, and their purity was assessed by SDS-PAGE with silver nitrate staining.²² Protein concentration was estimated by the Bradford assay.²³

Solid-Phase Binding Assays

Microtiter wells (Microtest 96-well flat-bottomed plate; Sarstedt, Nümbrecht, Germany) were incubated with 50 μ L of purified recombinant myocilin-HA (0.1 μ M) in coating buffer (50 mM NaCO₃, pH 9.6) for 12 hours at 4°C.^{24,25} A similar amount of BSA was also immobilized as a control. After coating, the supernatants were collected and analyzed by Western blot to check protein immobilization. The wells were blocked for 3 hours with 200 μ L of 10% BSA in coating buffer at 4°C. After the wells were washed three times with TBS-Tween (25 mM Tris-glycine [pH 7.5], 150 mM NaCl and 1% Tween-20), increasing concentrations (0–2.5 μ M) of purified recombinant proteins (myocilin and its N- and C-terminal fragments fused to the myc epitope at their C-terminal ends), dissolved in TBS-Tween containing 5% (wt/vol) BSA (50 μ L), were added to each well and incubated for 2 hours under gentle agitation (100 RPM) at room temperature. The wells were incubated with an anti-myc antibody (Santa Cruz), diluted at 1:300, for 2 hours at room temperature. A horseradish peroxidase-conjugated antibody against mouse IgG (Pierce) was diluted at 1:500. Colorimetric detection was performed by ELISA (1-Step Ultra TMB-ELISA; Pierce) for 30 minutes. The reaction was stopped by adding 50 μ L of 2 M H₂SO₄. Absorbance at 450 nm was determined in a microplate reader (Versa-Max; Molecular Devices, Sunnyvale, CA) in triplicate independent assays. Protein concentration was estimated by comparison with a calibration curve obtained with known amounts of immobilized proteins (SigmaStat 2.0 software; SPSS Science, Chicago, IL).

Far-Western Blot Analysis

Purified recombinant myocilin-HA and myocilin-myc (Western blot control) were separated by 8% SDS-PAGE under nonreducing conditions at room temperature and transferred to nitrocellulose membranes (Hybond ECL; Amersham).²⁶ After they were blot, the proteins were renatured in 10 $\times$ PBS for 4 hours at room temperature and blocked with 5% skimmed milk in PBS containing 0.01% Tween 20 for 90 minutes. The nitrocellulose membranes were incubated with each of the three conditioned culture media containing the different recombinant myocilin-myc versions for 12 hours at 4°C. Bound proteins were immunodetected by chemiluminescence as described in the Western blot section.

RESULTS

Role of Ionic and Disulfide Bonds in Myocilin Aggregation

To analyze the contribution of ionic interactions and disulfide bonds to the aggregation of myocilin, we treated the recombinant protein present in the culture medium of transiently transfected HEK-293T cells with increasing concentrations of NaCl (0.1–2.5 M) or β -mercaptoethanol (1–100 mM), at either room temperature or 95°C (Fig. 2). Myocilin in nontreated culture medium (control) showed a ladder pattern of aggregates, consisting of several bands larger than 118 kDa with the lower band probably corresponding to a myocilin dimer (Fig. 2, control lanes). Changes in ionic strength did not affect the

FIGURE 2. Effect of ionic strength or reduction of disulfide bonds on myocilin aggregation. A cDNA construct encoding myocilin carrying the myc epitope at the C-terminal end was used to transiently transfect 293-T cells. The culture medium was collected after 48 hours of expression, and 20- μ L aliquots were incubated with increasing concentrations of NaCl (0.1–2.5 M) or β -mercaptoethanol (1–100 mM) at either room temperature (RT) or 95°C for 10 minutes. Samples were analyzed by SDS-PAGE (4–15% gradient polyacrylamide) and Western blot with an anti-myc antibody. The control consisted of the culture medium containing recombinant myocilin in the absence of NaCl or β -mercaptoethanol incubated at either room temperature or at 95°C for 10 minutes.

myocilin aggregates at either room temperature or 95°C. Treatment of recombinant myocilin with β -mercaptoethanol at concentrations above 5 mM and at the two temperatures assayed completely disrupted the aggregates into myocilin monomers. Small variations in the electrophoretic mobility of the myocilin monomer, depending on the concentration of β -mercaptoethanol were observed (Fig. 2, β -ME lanes). They can be due to partial reduction of the intramolecular disulfide bridge between Cys-245 and Cys-433.^{15,27} These data indicate that ionic interactions do not mediate myocilin aggregation and that myocilin complexes are covalently linked by interchain disulfide bonds.

Effect of Proteolytic Processing on Myocilin Aggregation

Next, we analyzed the effect of myocilin cleavage on covalent aggregates. It has been shown that the processing of recombi-

FIGURE 3. Extracellular aggregation of recombinant myocilin transiently expressed for 24, 48, and 96 hours. A cDNA construct encoding myocilin carrying the myc epitope at the C-terminal was used to transiently transfect 293-T cells. The culture medium was collected after 24, 48, and 96 hours of expression, and 20- μ L aliquots were analyzed by SDS-PAGE (4–15% gradient polyacrylamide) and Western blot with an anti-myc antibody under either reducing (A) or nonreducing (B) conditions.

FIGURE 4. Extracellular aggregation of recombinant myocilin transiently expressed for 96 hours. A cDNA construct encoding myocilin carrying the HA and myc epitopes at their N- and C-terminal ends, respectively, was used to transiently transfect 293-T cells. The culture medium was collected after 96 hours of expression, and 20- μ L aliquots were analyzed by SDS-PAGE (10% polyacrylamide) and Western blot using anti-HA or anti-myc antibodies under either reducing (A) or nonreducing (B) conditions.

nant myocilin increases with the culture time.²¹ Thus, to achieve increasing levels of myocilin processing, cells transiently transfected with a cDNA encoding myocilin-myc were grown for 24, 48, and 96 hours, and the covalent aggregates in the culture medium were analyzed by gradient SDS-PAGE and Western immunoblot, under either reducing or nonreducing conditions (Fig. 3). As expected, the proteolytic cleavage increased with time, and recombinant myocilin in the culture medium was highly processed after 96 hours (Fig. 3A). The disulfide aggregates of myocilin detected under nonreducing

FIGURE 5. Western immunoblot analysis of myocilin present in bovine ocular tissues. Tissue extracts from the bovine trabecular meshwork, sclera, iris, ciliary body (40 μ g of total protein each), and aqueous humor (55 μ L) were prepared and fractionated by SDS-PAGE in 10% polyacrylamide gel and transferred to a nitrocellulose membrane. Conditioned medium (10 μ L) from transiently transfected HEK-293T cells expressing human recombinant myocilin was used as a control (Rec Hum Myoc). Myocilin was detected with the R14T polyclonal antibody. The electrophoretic mobility of full-length myocilin in the aqueous humor was affected by the elevated albumin concentration. *White and black arrowheads*: position of human and bovine myocilin, respectively. *Black arrow*: position of the 35-kDa myocilin band.

FIGURE 6. Purification of human recombinant myocilin and its N- and C-terminal fragments by Ni-chelating HPLC. Conditioned culture medium obtained from HEK-293T cells transiently transfected with cDNA constructs encoding myocilin-myc (A), the N-terminal-myc (B), or the C-terminal-myc fragment of myocilin (C) were processed and fractionated by Ni-chelating HPLC. Each culture medium (80 mL) was equilibrated in binding buffer and chromatographed. The column was eluted with the linear imidazole gradient indicated by the dotted line. Horizontal bars: the collected and pooled fractions. Insets: the recombinant proteins detected in the collected fractions by Western blot with an anti-myc antibody. CM, culture medium; BB binding buffer; NR nonretained.

conditions decreased as the proteolytic processing increased, whereas a parallel increment in the extracellular C-terminal fragment was noted (Fig. 3B). To analyze the aggregation of the N-terminal fragment, recombinant myocilin tagged with the HA and myc epitopes at its N- and C-terminal ends was transiently expressed in HEK-293T cells for 96 hours. Although the level of processing detected under reducing SDS-PAGE was similar to that obtained with the previous myocilin construct, the presence of the N-terminal fragment was significantly re-

duced in the culture medium, compared with the C-terminal fragment (Fig. 4A), because, as expected, it was retained intracellularly.²¹ Nonreducing 10% SDS-PAGE showed an aggregation pattern similar to that observed with the previous recombinant myocilin version, with the only difference that a band of approximately 85 kDa was detected with the anti-HA antibody (Fig. 4B). This band may correspond to aggregates of the N-terminal fragment. Overall, these data show that the proteolytic processing of myocilin reduces myocilin covalent aggre-

gates and indicate that the processed N-terminal fragment plays a limited role in the formation of extracellular aggregates.

Presence of C-terminal Myocilin Fragments in Ocular Bovine Tissues

In an effort to support that premise that the proteolytic processing of myocilin also occurs *in vivo*, particularly in the relevant tissues involved in IOP homeostasis (i.e., TM, CB, AH), we analyzed the presence of myocilin in different parts of the bovine eye by Western blot. We used the R14T antibody which recognizes an epitope located in the olfactomedin domain of myocilin (amino acids 272-285).^{5,15} Two major myocilin bands of approximately 66 and 35 kDa were detected in all the bovine tissues, except the sclera (Fig. 5). As expected, full-length bovine myocilin had an apparent molecular mass (66 kDa) that was higher than that of the human recombinant myocilin used as a positive control (55 kDa). The myocilin fragment present in bovine tissues was similar to that resulting from the proteolytic processing of human recombinant myocilin expressed in cells in culture (Fig. 5). These data provide further evidence of the existence of a physiologic proteolytic processing of myocilin in the inflow and outflow pathways.

Solid-Phase Binding Assays of Myocilin–Myocilin Interactions

The noncovalent interactions of myocilin and its processed fragments were investigated by solid-phase binding assays, for which different versions of recombinant myocilin were transiently expressed in HEK-293T cells and purified by Ni-chelating HPLC (Fig. 6). The HPLC fractions containing full-length myocilin-myc (17–19, Fig. 6A), the N-terminal-myc (9–11, Fig. 6B) or the C-terminal-myc fragment (10–15, Fig. 6C) were pooled and used for the assays. Full-length myocilin-HA was also purified by using a similar procedure (data not shown). The purity of the proteins, estimated by silver staining, was greater than 90% (data not shown). To analyze myocilin interactions, we coated the wells of microtiter plates with recombinant full-length myocilin-HA (0.1 μ M) and incubated them with increasing concentrations of each of the three recombinant proteins, tagged with the myc epitope at their C-terminal

FIGURE 7. Analysis of myocilin–myocilin interactions by solid-phase binding assays. Binding of recombinant full-length myocilin (●), the C-terminal fragment (▲) or the N-terminal fragment (□) to full-length myocilin-HA (0.1 μ M) immobilized in 96-well microtiter plates. The bound proteins tagged with the myc epitope at their C-terminal end were detected with an anti-myc antibody. The binding of full-length myocilin to immobilized BSA is depicted as a representative negative control (○). Error bars, SE of triplicate experiments.

FIGURE 8. Scatchard analysis of myocilin–myocilin interactions. The data obtained from the solid-phase binding assays (Fig. 7) were plotted in the Scatchard format. (●) Myocilin versus myocilin; (▲) myocilin-CT versus myocilin; (□) myocilin-NT versus myocilin; and (○) myocilin versus BSA.

ends. The three myocilin versions showed a concentration-dependent binding to immobilized myocilin (Fig. 7). Nonetheless, the interaction of the C-terminal fragment with myocilin was not saturable over the concentration range used, whereas myocilin and the N-terminal fragment showed a saturable binding to immobilized myocilin. These data indicate that noncovalent myocilin–myocilin interactions were mediated by their N-terminal regions, which is in agreement with previous studies.⁷ The Scatchard analysis of the data fitted a noncooperative single binding model (Fig. 8) and showed high-affinity for the interaction between full-length molecules ($K_d = 0.068 \mu$ M, Table 1). The affinity of the interaction between myocilin and the two recombinant fragments significantly decreased (Table 1). On the other hand, the binding capacity (B_{max}) of the interaction between the N-terminal fragment and myocilin was similar to that observed between the full-length molecules (Table 1). Nevertheless, B_{max} increased markedly for the interaction between the C-terminal fragment and myocilin. The increase could be explained by the reduction of steric hindrance owing to the lower molecular size of the C-terminal fragment and its limited capacity to aggregate under these conditions. As disulfide aggregates of recombinant full-length myocilin used in the assays were not disrupted, these data indicate that noncovalent interactions occur between myocilin oligomers.

Far-Western Analysis of Noncovalent Myocilin Interactions

We used Far-Western blot to confirm noncovalent interactions between recombinant myocilin aggregates. Full-length myocilin-myc and its N-terminal fragment showed specific noncovalent interactions with disulfide myocilin-HA aggregates (Figs. 9A, 9B). However, the C-terminal fragment failed to bind to myocilin under these conditions (Fig. 9C). Mock culture me-

TABLE 1. Apparent K_d and B_{max} Values of Myocilin-Myocilin Interactions Estimated by Scatchard Analysis

Interaction	K_d (μ M)	B_{max} (μ M)
Myocilin vs. myocilin	0.068	0.028
Myocilin C-T vs. myocilin	4.000	0.144
Myocilin N-T vs. myocilin	0.800	0.035

dium did not show any detectable interaction with myocilin (Fig. 9D). These data show the existence of specific noncovalent interactions between disulfide aggregates of recombinant myocilin and support that this type of binding takes place through the N-terminal region of the protein.

DISCUSSION

We have reported that recombinant myocilin is cleaved in the middle of the polypeptide chain by calpain II in the endoplasmic reticulum of cells in culture.^{15,21} The functional meaning of this proteolytic processing is unknown, but we have hypothesized that it might contribute to the regulation of its molecular interactions.^{15,21,28} In this study we found that the specific cleavage of recombinant myocilin reduces its extracellular covalent homoaggregates, linked by disulfide bonds, and increases the amount of the free extracellular C-terminal fragment. These data represent the first experimental evidence of the involvement of the proteolytic processing of myocilin in the regulation of myocilin aggregation. Our results indicate that ionic forces do not mediate myocilin aggregation, and they show that besides the covalent binding between myocilin monomers, myocilin complexes establish high-affinity, noncovalent interactions through their N-terminal regions. Myocilin-myocilin interactions within the N-terminal leucine zipper domain have been reported.⁷ These noncovalent interactions may be essential for myocilin's biological function. Of interest, the C-terminal fragment showed a very low-affinity binding to the full-length protein (4.0 μ M), suggesting that it remains free and available in the extracellular space to interact with other molecules. On the other hand, the affinity of the interaction between the N-terminal fragment and myocilin is also reduced (0.8 μ M). Our data suggest that the extracellular interactions of the N-terminal fragment probably plays a limited role in the function of the protein, because as we have shown here and in previous studies,^{15,21} the processed fragment is mainly retained intracellularly. Based on these data we hypothesize that covalent myocilin aggregates can specifically interact through noncovalent (hydrophobic) forces occurring at the N-terminal leucine zipper motif. These interactions may give rise to a complex and dynamic molecular myocilin network in extracellular compartments, such as the AH (Fig. 10A). According to this hypothesis, the activation of the proteolytic processing could reduce the myocilin network and simultaneously increase the free C-terminal olfactomedin fragment (Fig. 10B).

FIGURE 9. Far-Western blot analysis of noncovalent interactions between myocilin disulfide aggregates. Purified recombinant myocilin-HA and myocilin-myc (Western blot control) were subjected to SDS-PAGE (8% acrylamide) under nonreducing conditions, at room temperature. After electrophoresis, proteins were transferred to nitrocellulose membranes and probed with conditioned culture medium containing recombinant myocilin (A), the N-terminal fragment (B), the C-terminal fragment (C), or a mock culture medium (D). The three recombinant polypeptides used as probes were tagged with the myc epitope at their C-terminal ends. Bound proteins were detected by chemiluminescence using an anti-myc antibody.

FIGURE 10. Effect of myocilin proteolytic processing on self-aggregation. For simplicity the scheme shows a dimer, a trimer, and a tetramer linked by disulfide bonds (short black lines linking monomers). (A) According to this model when the proteolytic processing is switched off, all myocilin monomers form part of molecular aggregates. The N-terminal region of the protein (white rectangles) mediates covalent disulfide homoaggregation. Terminal myocilin monomers in the disulfide aggregates may also participate in noncovalent (hydrophobic) interactions (vertical dashed line) with other covalent aggregates through the leucine zipper motif (oblique dashed rectangles), giving rise to a dynamic molecular network. The antiparallel orientation of the monomers in the aggregates, which could alleviate steric hindrances between the globular olfactomedin domain (gray ovals) of the protein, is speculative. (B) Activation of the intracellular proteolytic processing reduces the amount of myocilin aggregates and increases the C-terminal fragment, which remains free as a monomer. The extracellular presence of the N-terminal fragment is reduced because it is mainly retained intracellularly.²¹

The leucine zipper and the first CC located in the N-terminal region of myocilin have also been shown to be involved in the adhesion of myocilin to the cell surface,²⁹ suggesting that the intricate extracellular myocilin network is linked to cells through the CC region of terminal myocilin molecules. Along this line, previous studies have reported that myocilin is present both in the AH and in the culture medium of transfected cells, but as high molecular weight homocomplexes^{7–9} which range in size from 116 kDa to more than 200 kDa,³⁰ and not as single monomers. In addition, large network structures composed of disulfide-linked oligomers are characteristic of other olfactomedin domain-containing proteins such as olfactomedin,^{31,32} amassin,³¹ photomedin,¹⁸ and noelin.³³

In a previous report, we identified C-terminal myocilin fragments in both the human and bovine CB and iris, as well as in human AH.¹⁵ In this study, we detected similar fragments in bovine TM and AH. C-terminal myocilin fragments have also been observed by other groups in the human TM³⁴ and transformed TM cells (TM5).³⁵ Moreover, myocilin fragments of approximately 22 kDa, which may correspond to the processed N-terminal domain are present in angle extracts of transgenic and wild-type mice.³⁶ Altogether these data support that myocilin undergoes *in vivo* proteolytic cleavage and therefore indicate that myocilin aggregation in these tissues may also be regulated by proteolysis. The identification of myocilin fragments in tissues involved in AH production and drainage suggests the possible involvement of the proteolytic cleavage of myocilin in modulating IOP. Further studies are needed to test these hypotheses.

In conclusion, this study showed for the first time that the proteolytic processing of recombinant myocilin regulates myocilin aggregation and highlights the importance of molecular interactions of myocilin in the extracellular space, thus providing an interesting clue to unravel the biological function of this enigmatic protein.

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