Myocilin Mutations Causing Glaucoma Inhibit the Intracellular Endoproteolytic Cleavage of Myocilin between Amino Acids Arg^{226} and Ile^{227}\textsuperscript{§§}$

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Myocilin is a secreted glycoprotein of unknown function that is ubiquitously expressed in many human organs, including the eye. Mutations in this protein produce glaucoma, a leading cause of blindness worldwide. To explore the biological role of myocilin and the pathogenesis of glaucoma, we have analyzed the expression of recombinant wild type and four representative pathogenic myocilin mutations (E323K, Q368X, P370L, and D380A) in transiently transfected cell lines derived from ocular and nonocular tissues. We found that wild type myocilin undergoes an intracellular endoproteolytic processing at the C terminus of Arg^{226}. This cleavage predicts the production of two fragments, one of 35 kDa containing the C-terminal olfactomedin-like domain, and another of 20 kDa containing the N-terminal leucine zipper-like domain. Here we have analyzed the 35-kDa processed fragment, and we have found that it is co-secreted with the nonprocessed protein. Western immunoblot analyses showed that human aqueous humor and some ocular tissues also contain the processed 35-kDa myocilin, indicating that the endoproteolytic cleavage occurs in vivo. Mutant myocilins accumulated in the endoplasmic reticulum of transfected cells as insoluble aggregates. Interestingly, the four pathogenic myocilins inhibited the endoproteolytic processing with varying efficiency. Furthermore, the mutation P370L, which produces the most severe glaucoma phenotype, also elicited the most potent endoproteolytic cleavage inhibition. We propose that the endoproteolytic processing might regulate the activity of myocilin and that the inhibition of the processing by pathogenic mutations impairs the normal role of myocilin.

Myocilin is a glycoprotein of unknown function, originally identified in cultured trabecular meshwork cells upon induction with glucocorticoids (1, 2). The cDNA was independently cloned from a subtracted ciliary body cDNA library (3, 4) and from a retinal cDNA library (5). Although the myocilin mRNA has been found to be ubiquitously expressed in many human tissues, its highest abundance appears to be restricted to tissues of the eye such as the iris, ciliary body, and trabecular meshwork (9–6). The gene encoding myocilin is currently referred to as MYOC. It consists of three different sized exons. The amino-terminal region of myocilin, encoded by exon 1, contains a peptide signal sequence (amino acids 1–32) and a leucine zipper-like motif composed of about 50 amino acid residues (amino acids 117–169) with periodic arginine and leucine repeats arranged along an α-helix (4). The finding of this amphipathic structure suggested that it might participate in molecular interactions (7). In fact, myocilin-myocilin interactions have been described to occur mainly within amino acids 117–166, in the region containing the leucine zipper-like domain (8). The central region of the protein (amino acids 203–245) is encoded by exon 2, and neither structural nor functional domains have been described in this location so far. The carboxyl-terminal half of myocilin, encoded by exon 3, is homologous to olfactomedin, an extracellular matrix protein of unknown role, that is abundant in the olfactory neuroepithelium (9). This domain contains a single disulfide bond connecting cysteine residues 245 and 433 (10). Interestingly enough, most mutations (missense) reported so far in the MYOC gene in glaucoma patients are heterozygous and are confined to exon 3 (4, 11–13).

Myocilin is intracellularly distributed in vesicles (14) and processed via the endoplasmic reticulum (ER)\textsuperscript{1} (15, 16). The protein is secreted to the aqueous humor of several species (17, 18) and into the culture medium of different cell lines as a doublet of nearly 55–57 kDa. The doublet is caused by glycosylation of both the myocilin variants and a slow nonglycosylated variant (19). Myocilin has been found to be ubiquitously expressed in many human tissues (10). Although the myocilin mRNA has been found to be ubiquitously expressed in many human tissues, its highest abundance appears to be restricted to tissues of the eye such as the iris, ciliary body, and trabecular meshwork (9–6). The gene encoding myocilin is currently referred to as MYOC. It consists of three different sized exons. The amino-terminal region of myocilin, encoded by exon 1, contains a peptide signal sequence (amino acids 1–32) and a leucine zipper-like motif composed of about 50 amino acid residues (amino acids 117–169) with periodic arginine and leucine repeats arranged along an α-helix (4). The finding of this amphipathic structure suggested that it might participate in molecular interactions (7). In fact, myocilin-myocilin interactions have been described to occur mainly within amino acids 117–166, in the region containing the leucine zipper-like domain (8). The central region of the protein (amino acids 203–245) is encoded by exon 2, and neither structural nor functional domains have been described in this location so far. The carboxyl-terminal half of myocilin, encoded by exon 3, is homologous to olfactomedin, an extracellular matrix protein of unknown role, that is abundant in the olfactory neuroepithelium (9). This domain contains a single disulfide bond connecting cysteine residues 245 and 433 (10). Interestingly enough, most mutations (missense) reported so far in the MYOC gene in glaucoma patients are heterozygous and are confined to exon 3 (4, 11–13).

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Glaucoma is a complex and genetically heterogeneous disease characterized by the progressive apoptotic death of retinal ganglion cells that leads to excavation of the optic nerve head and to visual field loss, eventually producing blindness (23, 24). Mutations in the MYOC gene cause autosomal dominant juvenile glaucoma (GLC1A) and a subset of adult onset primary open angle glaucoma (POAG). The best known risk factor associated with POAG is angle closure (25). The cataract surgery of a parent has also been associated with increased risk of POAG (26). The increased risk of POAG is associated with a number of ocular characteristics, such as a smaller iris angle (27), a shallower anterior chamber (28), and a larger corneal diameter (29). The specific mechanisms are not yet known.
associated with POAG is an increased intraocular pressure. The molecular pathway from the glaucoma genotype to the phenotype is not straightforward, and it probably involves a chain of subtle events. Over recent years, some of these events have been unraveled and have shown, for instance, that mutant forms of myocilin are not secreted in cultured cells; rather, they accumulate intracellularly as misfolded proteins. This leads to endoplasmic reticulum stress and to potential cytotoxicity (16, 18, 25, 26). Although certain molecular mechanisms such as homoallelic complementation, haploinsufficiency, or negative dominant effect have been proposed to explain the pathogenesis of myocilin glaucoma, most experimental evidence supports the gain of function theory (27–29).

Here we show for the first time that wild type myocilin is endoproteolytically processed, probably in the ER, and that pathogenic glaucoma mutations significantly inhibit this proteolytic processing. This work provides new insights not only into the physiological function of myocilin but also into the role it plays in the pathogenesis of POAG.

MATERIALS AND METHODS

Myocilin Constructs—Total RNA was extracted from 20 mg of frozen human skeletal muscle mechanically homogenized using Tri-Reagent (Sigma). Primers 5′-CGGAATTCTGAGGCTCTTCTGTGCAGG-3′ (primer 1) and 5′-GGGGATCCATTTGAGAGCTTGATGTC-3′ (primer 2) were employed to amplify human myocilin cDNA from the RNA preparation using the AccessQuick™ RT-PCR System (Promega). The sequence of primers was designed so that the PCR product incorporated EcoRI and BamHI restriction cleavage sites. The obtained cDNA, which contained the full-length myocilin sequence, including its signal peptide, was cloned into the EcoRI-BamHI sites of the mammalian expression vector pcDNA3.1 (Invitrogen). This vector was previously adapted by inserting coding sequences for Myc and His epitopes or green fluorescent protein (GFP) in the BamHI-HindIII sites. DNA from the wild type myocilin clone was nonrecombinant containing Myc-His epitopes was used as a template to produce different point mutations with the QuikChange site-directed mutagenesis kit (Stratagene). The specific PCR primers used for mutagenesis were as follows: 5′-CTTATAGGCCTAGAAGGAGCCGGTCTG-3′ and 5′-CAACGCACACTTTTCAGAAGGCTAG-3′ for E323K, 5′-CCAGGACAGTCTCTGTTTCAGCTAG-3′ and 5′-GGCCACCCCAAGAATACAGAAGCTTTCCG-3′ for P280L, and 5′-GGCTACACCGGATTGCGCTTTGAGGCTAG-3′ and 5′-CATCCACAGCACCGGGACTCAGTTGTTGACCTG-3′ for D380A. The truncated version of myocilin Q368X was also produced by PCR using the following primers: 5′-CGGAATTCTGAGGCTCTTCTGTGCAGG-3′ and 5′-GGGGATCCCTTGCAGGCTAG-3′. The mutated cDNAs were subcloned into the pCDNA3.1 vector already containing the Myc-His epitope using the expression vector pcDNA3.1 (+) adapted as described above. To prepare the deletion construct R226-E230del, two PCR fragments were first amplified from each side of the deletion using as template the full-length myocilin cDNA cloned as described above. The 5′ portion of this construct was amplified using primer 1 and the deletion-specific sense primer 5′-GG- TAATCTGAATGTCACCTGAGATAGCTGCTGAGGAGGCTATCAGTTGACG-3′. The 3′ portion was amplified using the deletion-specific sense primer 5′-GG- TAATCTGAATGTCACCTGAGATAGCTGCTGAGGAGGCTATCAGTTGACG-3′ and primer 2. The primary PCR products were mixed and used as a template in a secondary PCR with primers 1 and 2. The PCR fragment containing the deleted region was directly cloned into the EcoRI-BamHI sites of the pcDNA3.1-Myc-His vector. All of the DNA constructs obtained were sequenced to confirm both the correct insertion and absence of undesirable mutations.

Tissues and Cell Lines—Bovine and human eyes were obtained from a local slaughterhouse or from human cadavers within 24 h after enucleation through the National Disease Research Interchange (Philadelphia, PA), respectively. Human aqueous humor samples were collected from patients undergoing intraocular surgery after obtaining informed consent. Investigation of the human subjects was approved by the Human Subjects Committee of Yale University and followed the tenets of the Declaration of Helsinki, as far as it applies. Eyes were microdissected from the posterior pole, both the vitreous and lens were removed, and the ciliary body and iris were microdissected. Homogenization of ciliary body and iris was performed by mechanical grinding with liquid nitrogen using a mortar and pestle. Pulverized frozen tissue was suspended in 300 μl of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% (v/v) Triton X-100, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 mM Na3VO4, and 1 mM NaF), vortexed for 30 s at maximum speed, and incubated for 30 min on ice. The lysates were centrifuged at 20,000 × g for 5 min at 4 °C. Supernatants were stored at −80 °C until used.

Western Blotting and Antibodies—Analytical 10% polyacrylamide slab gel electrophoresis in the presence of SDS was performed as reported by Laemmli (31), using the Mini-PROTEAN III gel electrophoresis system (Bio-Rad). Gels were stained with Coomassie Brilliant Blue R250 (32). For Western blot analysis aliquots of culture medium, intracellular fractions of cultured cells (both soluble and insoluble) or pellets (cellular insoluble fraction) were carefully separated from the pellets using a commercial mixture of proteinase inhibitors (Pierce). Collected cells were vortexed for 30 s at maximum speed, incubated for 30 min on ice, and sonicated for 10 s (cycle 0.5 s). Cells lysates were centrifuged at 20,000 × g for 5 min at 4 °C. The supernatants (cellular soluble fraction) were separated from the cellular insoluble fraction. Both cellular fractions were stored at −80 °C until used. The efficiency of transfections was estimated in cells transiently transfected with a CDNA construct encoding myocilin-GFP, by counting the number of GFP-positive cells in a total of 105 cells in four randomly selected areas per dish. The average transfection efficiency was 20–25%. As negative control, the different cell lines were transfected with 0.5 μg of pcDNA3.1 vector.

Endoproteolytic Processing of Myocilin—Following transfection with different myocilin constructs, cells were processed as described above. Adherent cells were washed twice with 1 ml of Dulbecco’s phosphate-buffered saline (150 mM NaCl, 3 mM KCl, 1 mM KH2PO4, 6 mM Na2HPO4, 0.5 mM MgCl2, 1 mM CaCl2, pH 7.2), followed by an addition of 200 μl of lysis buffer containing proteinase inhibitors. Collected cells were vortexed for 30 s at maximum speed, incubated for 30 min on ice, and sonicated for 10 s (cycle 0.5 s). Cells lysates were centrifuged at 20,000 × g for 5 min at 4 °C. The supernatants (cellular soluble fraction) were separated from the pellets (cellular insoluble fraction). Both cellular fractions were stored at −80 °C until used. The efficiency of transfections was estimated in cells transiently transfected with a CDNA construct encoding myocilin-GFP, by counting the number of GFP-positive cells in a total of 105 cells in four randomly selected areas per dish. The average transfection efficiency was 20–25%. As negative control, the different cell lines were transfected with 0.5 μg of pcDNA3.1 vector.

Cell Transfections—Cells growing in 6-well plates at 70–80% cell confluence were transiently transfected with 0.5–1.0 μg of plasmid soluble fraction the Supernatant (Supernatant) according to the manufacturer’s instructions. In order to avoid proteolytic degradation of secreted recombinant myocilin, a commercial mixture of proteinase inhibitors for specific use in culture media (Sigma) was used. Forty-eight h after transfection, the culture medium was collected. To remove cellular debris from the collected culture medium, samples were centrifuged at 5,000 × g for 5 min. The supernatant was stored at −80 °C until used. Adherent cells were washed twice with 1 ml of Dulbecco’s phosphate-buffered saline (150 mM NaCl, 3 mM KCl, 1 mM KH2PO4, 6 mM Na2HPO4, 0.5 mM MgCl2, 1 mM CaCl2, pH 7.2), followed by an addition of 200 μl of lysis buffer containing proteinase inhibitors. Collected cells were vortexed for 30 s at maximum speed, incubated for 30 min on ice, and sonicated for 10 s (cycle 0.5 s). Cells lysates were centrifuged at 20,000 × g for 5 min at 4 °C. The supernatants (cellular soluble fraction) were separated from the cellular insoluble fraction. Both cellular fractions were stored at −80 °C until used. The efficiency of transfections was estimated in cells transiently transfected with a CDNA construct encoding myocilin-GFP, by counting the number of GFP-positive cells in a total of 105 cells in four randomly selected areas per dish. The average transfection efficiency was 20–25%. As negative control, the different cell lines were transfected with 0.5 μg of pcDNA3.1 vector.

Nickel-chelating Chromatography of Recombinant Myocilin—Recombinant wild type myocilin was isolated by nickel-chelating chromatography using a Hi-Trap Chelating HP 1 ml column (Amersham Biosciences). Two ml of culture medium from transiently transfected 293T cells were equilibrated in binding buffer (20 mM Tris-HCl, pH 7.9, 0.5 mM NaCl) containing 5 mM imidazole, using Amicon Ultra-4 Centrifugal
Western immunoblot at 6, 12, 24, and 48 h after transfection by using the anti-Myc 9E10 antibody. The most appropriate production of wild type myocilin was accomplished at 48 h post-transfection with 0.5–1.0 μg of DNA (data not shown). These conditions were used in subsequent experiments.

Western blot analysis employing the anti-myocilin R14T antibody, which binds to a peptide located at the beginning of the olfactomedin-like domain (Supplementary Fig. 1), showed that myocilin was present in the culture medium of the four transfected cell lines (293T, COS1, 59HIVs, 26HCMSv cells). Forty-eight h after transfection, myocilin was analyzed in the culture medium (5–20 μg) and in the intracellular fraction (20 μg of total protein) by SDS-PAGE in 10% polyacrylamide gels. A sample of human aqueous humor (5 μl) and whole tissue extracts (5–20 μg of total protein) from human and bovine iris and ciliary body, prepared as described under “Materials and Methods,” were also analyzed. Detection was carried out with the R14T anti-myocilin polyclonal antibody. Negative controls consisted of culture medium and cell lysates from 26HCMSv cells transfected with the nonrecombinant vector pcDNA3.1-Myc-His (−) lanes). The broad shadowed signal of around 60 kDa, indicated with an asterisk, is originated by weak nonspecific binding of the antibody to albumin. Film exposure was for 30 s. The arrows indicate the position of the 35-kDa myocilin band. AH, aqueous humor; B, bovine; CB, ciliary body; H, human. The intracellular fraction of 293T cells from A was overexposed to allow for the detection of the 35-kDa myocilin band (see the arrow). Film exposure was for 5 min. C, affinity purification of myocilin from a human ciliary body extract. A lysate of human ciliary body (1 mg of protein) was applied to a column of AminoLink Plus resin (Pierce) linked to the C21A anti-myocilin. Fifty μl of the retained fraction (R.F.) were then fractionated by SDS-PAGE and assayed by Western immunoblot with the R14T anti-myocilin antibody. Five μl of whole ciliary body tissue extract were applied to the column as control. The arrowhead indicates the position of the 55-kDa myocilin band. D, to assess antibody specificity, aliquots (5 μl) of culture medium from COS1 cells transfected with the DNA construct encoding Myc-tagged wild type myocilin (Supplementary Fig. 1) were analyzed by Western immunoblot (lanes 1 and 2). Detection was performed with the C21A antibody (left) or with the anti-Myc 9E10 monoclonal antibody (Santa Cruz Biotechnology) (right). Negative controls consisted of culture medium (5 μl) from COS1 cells transfected with the nonrecombinant vector pcDNA3.1-Myc-His (−) lanes). Film exposure was for 30 s. E, quantitation by densitometry of the low molecular mass myocilin band (35 kDa) detected in different samples from A. Values are expressed as a percentage of total detected myocilin (55 + 35-kDa bands). The error bars represent the S.E. of triplicate experiments.
FIG. 2. Purification of myocilin secreted by transiently transfected 293T cells. The DNA construct encoding wild type myocilin (Supplementary Fig. 1) was transfected into 293T cells. The culture medium was collected 48 h after transfection and equilibrated in binding buffer as described under “Materials and Methods.” Two ml of sample were loaded into a Hi-Trap Chelating HP column, and then the column was stepwise eluted with 5 ml of binding buffer and 5 ml of binding buffer containing 75 mM imidazole. The retained molecules were eluted with 5 ml of binding buffer containing 1 M imidazole. Three μl from each chromatographic fraction were analyzed by SDS-PAGE in a 10% polyacrylamide gel. A, proteins were detected by Coomassie Blue staining. As a control, 10 μl of the sample loaded into the chromatographic column were also analyzed (left lane). B, a duplicated gel of that shown in A was analyzed by Western immunoblot using the anti-Myc 9E10 monoclonal antibody to detect recombinant myocilin. B.B., binding buffer; imid, imidazole. C, aliquots of 15 μl of culture medium were either treated with loading buffer containing β-mercaptoethanol and boiled for 5 min (reduced) or were treated with loading buffer without β-mercaptoethanol and without boiling (nonreduced). Detection was performed with the anti-Myc 9E10 monoclonal antibody. The arrows indicate the position of the 35-kDa myocilin band. The arrowhead indicates a 35-kDa high mobility band detected under nonreducing electrophoresis.

Fig. 1B (see arrow), where the overexposed intracellular fraction of 293T cells is shown as a representative example.

The 35-kDa band was also seen in human aqueous humor as well as in bovine iris and ciliary body whole extracts (Fig. 1A, H, AH and Tissues lanes; see arrow). Nonetheless, it was detected in whole extracts from human iris and ciliary body only after overexposing the blots (data not shown). Full-length myocilin present in bovine tissues displayed an apparent molecular weight higher than that of myocilin visualized in human tissues or in cell lines. This is in accordance with previous studies (36). Since lengths of human and bovine myocilin polypeptide chains are approximately the same, this difference can be explained by distinct posttranslational modifications of the protein in these two species.

The specificity of the anti-myocilin R14T antibody was shown by the absence of bands in control samples of culture medium and cell lysates from the four cell lines transfected with the nonrecombinant vector. Fig. 1A (→ lanes) shows a representative result obtained with 2BHCMSv cells. Moreover, in previous experiments, we determined that the use of preimmune serum or preincubation of the antibody with the immunizing peptide in Western blots did not recognize any band (data not shown), further supporting the specificity of the R14T antibody.

To confirm that the human ciliary body produces the myocilin fragment, we isolated this polypeptide by affinity chromatography. The soluble protein from a ciliary body extract was fractionated using the resin-coupled C21A anti-myocilin antibody. Western immunoblot analysis with the R14T anti-myocilin antibody showed that the bound fraction contained the 35-kDa band (Fig. 1C, lane R.F.; see arrowhead and arrow). Since R14T and C21A antibodies recognize epitopes placed at the beginning and at the end of the olfactomedin-like domain, respectively, these data show that the 35-kDa band corresponds to a C-terminal myocilin fragment, which contains the complete olfactomedin-like domain. Both the culture medium and lysis buffer incorporated a mixture of proteasome inhibitors as indicated under “Materials and Methods.” Therefore, a nonspecific proteolytic cleavage of myocilin during either myocilin expression or sample preparation is unlikely.

The specificity of the anti-myocilin C21A antibody was assessed by Western blot on recombinant Myc-tagged myocilin expressed in COS1 cells. The antibody stained only two bands at 55 and 35 kDa (Fig. 1D, lane 1). This profile was identical to the one obtained with both the well characterized anti-Myc 9E10 monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (Fig. 1D, lane 2) and the anti-myocilin antibody R14T (Fig. 1A, culture medium lanes). No bands were detected in control samples of culture medium (Fig. 1A and D; see → lanes). Altogether, these data demonstrate the specificity of the antibodies.

The percentage of the secreted myocilin fragment was calculated by densitometry as the ratio of the 35-kDa band volume (intensity of the pixels × mm²) to the total volume of myocilin bands (55 + 35 kDa). Nearly 70% of myocilin secreted by 293T cells corresponded to the 35-kDa fragment, whereas it ranged from 30 to 40% in the other three cell lines (Fig. 1E). The fragment represented around 12, 60, and 65% of total myocilin in human aqueous humor, bovine iris, and CB, respectively (Fig. 1E).

Isolation and Characterization of the 35-kDa Myocilin Fragment—In order to characterize the 35-kDa myocilin fragment, we took advantage of the His-tag present in the C terminus of recombinant myocilin. Secreted myocilin was purified from the culture medium of transiently transfected 293T cells by nickel-chelating chromatography, as described under “Materials and Methods.” The total protein content of each chromatographic fraction was estimated by SDS-PAGE with Coomassie Blue staining (Fig. 2A), whereas the presence of recombinant myocilin was determined in a duplicated gel, by Western immunoblot using the anti-Myc antibody (Fig. 2B). Myocilin signals of 55 and 35 kDa eluted with 1 M imidazole (Fig. 2B). The 35-kDa fragment present in this fraction (see arrow in Fig. 2A) was further isolated by SDS-PAGE and analyzed by amino acid microsequencing and MALDI-TOF. The following N-terminal amino acid sequence was obtained: Ile-Leu-Lys-Glu-Ser-Pro.

Endoproteolytic Processing of Myocilin
between full-length myocilin and its C-terminal fragment, samples of culture medium from transfected 293T cells were analyzed by SDS-PAGE under reducing and nonreducing conditions. The nonreduced and nonboiled sample showed two bands of around 120 and 180 kDa and an intense signal of around 35 kDa (Fig. 2C). The high molecular mass bands could represent dimmers or trimmers of myocilin, whereas the lower band matches with the C-terminal fragment of the protein. Interestingly, this band in the nonreduced sample slightly increased its electrophoretic mobility when compared with the reduced sample (Fig. 2C; see arrows). This change suggests the existence of an intramolecular disulfide bridge between the only two cysteines of the C-terminal region of myocilin (Cys433 and Cys435). In summary, these results show that full-length myocilin and the 35-kDa fragment are not linked by disulfide bonds, and they point out that the two polypeptides do not interact through ionic bonds, although interaction by hydrophobic forces cannot be ruled out.

To further confirm the specificity of the cleavage site, we deleted a highly conserved pentapeptide (Arg226-Ile-Leu-Lys-Glu230), which (i) encompasses the two amino acids directly involved in the proteolytic processing (Arg226-Ile227) and (ii) matches the consensus sequence of the subtilisin/kexin isozyme-1 cleavage site (see below). We studied the processing of this construct in transiently transfected 293T cells. As can be seen in Fig. 4, 48 h after transfection, the R226-E230del myocilin mutant was produced and secreted to the culture medium exclusively as the full-length form. These data show the specificity of the identified cleavage site.

Treatment of Myocilin-transfected 293T Cells with BFA—To further confirm the intracellular cleavage of myocilin, 293T transfected cells were treated with the protein secretion inhibitor BFA (1 µg/ml). As Fig. 5 shows, BFA blocked the secretion of myocilin into the culture medium and induced a remarkably accumulation of the 35-kDa fragment in the intracellular soluble fraction. Since BFA inhibits protein transport from the ER to the Golgi and between the Golgi cisternae, this result suggests that the 35 kDa fragment is intracellularly produced in the ER. Cell lysates were prepared in the presence of a mixture of proteinase inhibitors as previously mentioned; therefore, a nonspecific proteolytic degradation of myocilin occurring while cell lysates were prepared is unlikely.

Bioinformatic Analysis of the Polypeptide Region of Myocilin Containing the Cleavage Site—We searched for domains around the cleavage site of myocilin in the collection of protein families and domains incorporated in the Pfam data base (34). We also searched for putative sequences recognized by different proteolytic enzymes in the ELM data base (35). The predicted domain, called Pfam-B_13130, ranging from amino acid 185 to 245. This domain is mostly encoded by exon 2 and locates within a conserved region of mammalian myocilins. It also contains a stretch of amino acids predicted to fold in α-helix (Asp208-Val222) (Fig. 6A). The amino acid residue placed at the N-terminal part of the cleavage point is Arg in the human myocilin, yet it is Gln in other species. Since we have shown that bovine myocilin also undergoes proteolytic cleavage, probably in the same place, the residue in this position does not appear to play a relevant role in the processing.

We also searched for putative sequences recognized by different proteolytic enzymes in the ELM data base (35). The
Both the amino acids encoded by exon 2 and the region predicted to fold Arg226 inside the region encoded by exon 2 is shown. Note that the teolytic processing of myocilin. The location of the cleavage site at indicates the position of the 35-kDa myocilin band.

Western immunoblot of myocilin expressed in transiently transfected 293T cells treated with the protein secretion inhibitor BFA. One μg of the DNA construct encoding wild type myocilin (Supplementary Fig. 1) was transiently transfected into 293T cells and then cultured for 24 h with Dulbecco’s modified Eagle’s medium containing BFA at a final concentration of 1 μg/ml. The culture medium (20 μl) and the intracellular fraction (20 μg of total protein) were processed as indicated under “Materials and Methods.” Detection was performed with the anti-Myc 9E10 monoclonal antibody. The arrow indicates the position of the 35-kDa myocilin band.

Expression of Mutant Pathogenic Myocilins in Cultured Cell Lines—With the aim of analyzing the possible effect of myocilin pathogenic mutations in the described processing, we expressed four representative glaucoma mutations in COS1, 293T, and 26Cmv cell lines. We selected the nonsense mutation Q368X as well as the missense mutations E323K, P370L, and D380A (Supplementary Fig. 1).

The three missense and the nonsense myocilin mutants accumulated in the insoluble cellular fraction as 55- and 40-kDa molecules, respectively (Fig. 7, A–C, insoluble cellular fraction lanes), in agreement with previous works (16, 18, 25). Interestingly, a fraction of mutant myocilins were also detected as intracellular soluble molecules (Fig. 7, A–C, soluble cellular fraction lanes). It is worth noting that under these conditions, the 35-kDa myocilin fragment was not detected in any sample from cells expressing mutant myocilins (Fig. 7, A–C; see the arrow on the right), suggesting that the three missense pathogenic mutations inhibit the intracellular proteolytic processing of the olfactomedin-like domain. In contrast, and as shown before, the wild type myocilin fragment was present in both the culture medium and the intracellular soluble fraction in control transfections. These experiments were repeated at least three times in each cell line, and the results were consistent.

Expression of Mutant Pathogenic Myocilins in Cultured Cell Lines—Treated with Monensin—To confirm the inhibitory effect of pathogenic mutations on the intracellular processing of myocilin, we blocked protein secretion of 293T transfected cells with monensin (0.32 μg/ml). This cell line was selected, since it showed the highest efficiency of endoproteolytic cleavage site (Fig. 6B).

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A population of cells transfected with the nonrecombinant vector pcDNA3.1-Myc-His (Fig. 8A, Insoluble cellular fraction lanes). Around 80% of intracellular soluble wild type myocilin corresponded to the 35-kDa processed fragment (positive control) (Fig. 8B), but in contrast, the intracellular missense myocilin fragment ranged from 50% (E323K and D380A) to 10% (P370L) (Fig. 8B). Interestingly, these data revealed that the myocilin mutation P370L, responsible for the most severe glaucoma phenotype, produced the highest inhibition of the endoproteolytic processing. Cells expressing the myocilin mutation Q368X only showed the 40-kDa doublet (Fig. 8A), indicating that its truncated olfactomedin-like domain is not cleaved. These results support that glaucoma mutations inhibit the intracellular processing of myocilin with varying efficiency.

Subcellular Localization of Wild Type and Pathogenic Mutations in Cultured Cell Lines—We analyzed the microscopic distribution of wild type and mutant pathogenic myocilins in transiently transfected 293T and 26HCMsv cells using DNA constructs encoding myocilins tagged with GFP in their C terminus. This tagging did not affect their previously characterized secretory pattern as determined by Western immunoblot using a monoclonal anti-GFP antibody (data not shown). This is in accordance with previous reports (38, 39).

The analysis of GFP fluorescence revealed that wild type myocilin was distributed in vesicular structures and in a discrete reticular network located around the nucleus and cytoplasm in 293T cells (Fig. 9A). Moreover, intense fluorescence was detected in the perinuclear region, showing a Golgi-like staining (Fig. 9B). These signals were similar to those produced by specific fluorescent markers for the ER and Golgi apparatus (Fig. 9, G and H). No staining of other intracellular structures was detected. These results clearly show that wild type myocilin-GFP locates in the secretory pathway, thus supporting the notion that it is proteolytically processed in the ER. Similar results were obtained with the 26HCMsv cell line (data not shown). The analysis of GFP fluorescence from cells expressing the four pathogenic myocilin mutations also localized in the perinuclear region showing a pattern that suggested ER staining, but it was more diffuse than that observed with wild type myocilin (Fig. 9, C–F). Intense granular structures in the cytoplasm of cells expressing pathogenic myocilins were often observed, suggesting that most of these molecules aggregate and accumulate in the ER (data not shown). These results show that both missense and nonsense mutant myocilins mainly accumulate as insoluble aggregates inside the ER, in agreement with previous reports (26, 39). We also detected weak staining of vesicular structures in cells expressing mutant myocilins (data not shown), indicating the existence of a small fraction of soluble molecules, which escape precipitation in the ER. These data are also in accordance with our Western immunoblot analyses. Similar results were obtained with 26HCMsv cells (data not shown).

**DISCUSSION**

The present study clearly shows that ocular and nonocular cultured cell lines co-secrete full-length myocilin and a fragment of 35 kDa, which contains the olfactomedin-like C-terminal domain of the protein. This 35-kDa fragment has also been identified in human aqueous humor and whole tissue extracts from the human and bovine iris and CB, suggesting that it originates by a physiological processing. We have isolated and characterized this fragment by Edman sequencing and MALDI-TOF peptide mass fingerprinting. These analyses revealed that the fragment starts at Ile227, and they confirmed that it contains the complete olfactomedin-like domain of myocilin. In addition, we have shown that it probably arises intracellularly in the ER. Overall, these data show for the first time that myocilin undergoes a specific and intracellular endoproteolytic cleavage at the C-terminal end of Arg227, a site placed inside the region encoded by the exon 2 of the MYOC gene. The described processing leads to a separation of the C-terminal olfactomedin-like domain from the N-terminal leucine-zipper-like domain and predicts the formation of two fragments of about 35 and 20 kDa, respectively (Fig. 6B). The antibodies used in this study recognize epitopes located in the C-terminal region of the protein; therefore, they did not allow the putative 20-kDa N-terminal processed fragment to be detected. Studies are under way to explore whether the processed N-terminal fragment is secreted. Our data also suggest that the C-terminal fragment is rapidly secreted after cleavage, since we were able to clearly detect it in the intracellular medium of transfected cell lines only when protein secretion was inhibited.

Several studies have reported the existence of myocilin fragments of ~30–35 kDa in samples of recombinant myocilin expressed in cell lines and in tissue extracts (36, 40, 42). These fragments were usually interpreted as products of nonspecific myocilin proteolysis. Four findings sustain that the 35-kDa myocilin fragment is not a product of nonspecific degradation: (i) general proteinase inhibitors do not affect its production, as Goldwich et al. also observed (40); (ii) deletion of the cleavage site inhibits the production of the 35-kDa fragment; (iii) the
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**FIG. 9.** Subcellular distribution of human wild type myocilin-GFP and different mutants found in glaucoma patients in transiently transfected 293T cells. 1 μg of DNA constructs encoding GFP-tagged versions of wild type myocilin (A and B) and mutant myocilin forms E323K (C), Q368X (D), P370L (E), and D380A (F) were transfected into 293T cells. Both the ER and Golgi apparatus were visualized by transfecting cells with DNA constructs encoding either GFP-tagged versions of the signal peptide of calreticulin (G) or the first 80 N-terminal amino acids of galactosyl-transferase (H), respectively. These two constructs are specific fluorescent markers for both the Golgi apparatus and ER, respectively. Wild type myocilin was mainly detected in structures compatible with the Golgi apparatus and secretory vesicles. Note that the four mutant versions accumulated in the ER. The *asterisks* indicate the location of the Golgi apparatus. The *arrows* indicate the position of the secretory vesicles. Original magnification was x1600.

35-kDa fragment originates intracellularly, probably in the ER and; (iv) different pathogenic mutations also inhibit the endoproteolytic cleavage. Interestingly, one such reported fragment was produced by 293 EBNA cells as a doublet, and it was determined that it matched the C-terminal region of myocilin, starting at the amino acid residue Leu215 (40), in a place close to the cleavage site reported here. These data raise the possibility that proteolysis might occur at other nearby sites. In accordance with this observation, we have detected minor myocilin bands of around 35 kDa in overexposed Western immunoblots (data not shown).

Interestingly, the proteolytic cleavage takes place at Arg226, an amino acid residue located in the central part of myocilin, inside a region encoded by the MYOC exon 2. This part of the protein joins the N- and C-terminal domains in the unprocessed molecule. Exon 2 encodes a polypeptide of 42 amino acids and does not contain membrane-spanning regions, thus fulfilling the requirements of interdomain linker regions (43). In addition, amino acids encoded by exon 2 belong to the Pfam-B_13130 domain identified by a bioinformatic analysis in the Pfam database. Based on these data, we propose that the MYOC exon 2 encodes a putative linker domain and that it is involved in the endoproteolytic processing of myocilin (Fig. 6B). Mukhopadhyya et al. (44) also suggested that this exon encodes a linker domain.

The finding of the endoproteolytic processing raises the possibility that it might regulate the extracellular activity of myocilin on a post-translational level, as has been described for many secretory proteins (45, 46). It has been suggested that myocilin might play a role in the extracellular matrix structure and that it might contribute to regulate the normal aqueous humor outflow. Hence, we speculate that controlled changes in the proportion of processed myocilin might contribute to regulate the normal trabecular meshwork structure by the establishment of intermolecular interactions with other extracellular matrix molecules, therefore contributing to modulate the aqueous humor outflow and the intraocular pressure.

The results presented here clearly show that myocilin is a secretory protein that is intracellularly located in the ER, the Golgi apparatus, and in secretory vesicles. Several studies have analyzed the subcellular location of myocilin and have yielded diverse results with co-localizations such as microtubules (47), mitochondria (48), and secretory pathway (21, 39). No evidence of the myocilin location being outside the secretory pathway was obtained, which is in accordance with O’Brien et al. (21) and Sohn et al. (39).

We extended the study to research the effect of four representative myocilin pathogenic mutations on the endoproteolytic processing of myocilin. We selected the nonsense mutation Q368X, since it is the most prevalent in POAG patients. The missense mutation P370L was chosen, since it is responsible for the most severe glaucoma phenotype. E323K and D380A mutations were studied because of their strong association with familial glaucoma (37, 41, 49). In accordance with previous studies, we found that myocilin mutations promoted the accumulation of the protein in the ER as insoluble aggregates (16, 18, 25). However, a fraction of mutant molecules was clearly detected in the cellular soluble fraction, raising the possibility that they are secreted. Further studies are required to clarify this point.

Pathogenic mutations studied here were found to inhibit the endoproteolytic processing of myocilin with varying efficiency, providing an interesting genotype-phenotype correlation. The myocilin mutation P370L, responsible for the most severe glaucoma phenotype, produces the highest inhibition. On the other hand, the suppression of the endoproteolytic cleavage by two mutations that cause less severe and comparable phenotypes, E323K and D380A, was also similar and less intense. Although the relationship between inhibition of myocilin processing and glaucoma severity is unknown, it provides a new clue for further investigations. The Q368X myocilin mutation represents a particular case in glaucoma pathogenesis, since it produces a truncated protein that lacks the C-terminal half of the olfactomedin-like domain, and it is unlikely to undergo cleavage at Arg226. This variation could explain why this myocilin mutation shows a milder phenotype with a low penetrance despite the fact that it is prevalent. In this case, it might be that only cytotoxicity, another pathogenic mechanism that is proposed to play a role in glaucoma, contributes to the phenotype. Previous studies have also suggested that the Q368X mutation may represent a special case, since premature termination mutations may lead to a decreased mutant protein production due to the degradation of unstable mRNA (20). However, no reduced expression of the truncated myocilin has been observed in our...
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experiments when compared with the three missense mutants. Since the analyzed myocilin mutations do not directly affect the cleavage site, they could inhibit the proteolytic processing as the result of a structural change that alters binding of the endoprotease to its substrate. Further investigations are required to determine the exact inhibitory mechanism.

The finding that myocilin undergoes a specific endoproteolytic processing in the ER and that it is inhibited by pathogenic mutations provides new opportunities to unravel both its enigmatic role in normal tissues and in glaucoma pathogenesis.

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