Extracellular Trafficking of Myocilin in Human Trabecular Meshwork Cells*§

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Myocilin (MYOC) is a protein with a broad expression pattern, but unknown function. MYOC associates with intracellular structures that are consistent with secretory vesicles, however, in most cell types studied, MYOC is limited to the intracellular compartment. In the trabecular meshwork, MYOC associates with intracellular vesicles, but is also found in the extracellular space. The purpose of the present study was to better understand the mechanism of extracellular transport of MYOC in trabecular meshwork cells. Using a biochemical approach, we found that MYOC localizes intracellularly to both the cytosolic and particulate fractions. When intracellular membranes were separated over a linear sucrose gradient, MYOC equilibrated in a fraction less dense than traditional secretory vesicles and lysosomes. In pulse-labeling experiments that followed nascent MYOC over time, the characteristic doublet observed for MYOC by SDS-PAGE did not change, even in the presence of brefeldin A; indicating that MYOC is not glycosylated and is not released via a traditional secretory mechanism. When conditioned media from human trabecular meshwork cells were examined, both native and recombinant MYOC associated with an extracellular membrane population having biochemical characteristics of exosomes, and containing the major histocompatibility complex class II antigen, HLA-DR. The association of MYOC with exosome-like membranes appeared to be specific, on the extracellular face, and reversible. Taken together, data suggest that MYOC appears in the extracellular space of trabecular meshwork cells by an unconventional mechanism, likely associated with exosome-like vesicles.

Myocilin (MYOC), also known as trabecular meshwork inducible glucocorticoid response protein, is an acidic 504-amino acid protein. Structurally, MYOC contains at least two folding domains, an N-terminal coiled-coil and a C-terminal globular domain with significant homology to an olfactomedin module present in several different proteins (1, 2). Mammalian proteins with the olfactomedin module localize to different compartments of the secretory pathway, although little is known about the function of these proteins or the olfactomedin module (3–10).

Despite a broad expression pattern (1, 2, 7, 11–13), the function of MYOC remains unknown and its cellular distribution ambiguous. For example, MYOC has been observed in various cell types to associate with structures that are part of the secretory pathway, including endoplasmic reticulum, Golgi apparatus, and intracellular vesicles. Conversely, MYOC has also been reported to associate with mitochondria and cytoplasmic filaments (11, 14–17). Even more unusual, MYOC appears to be secreted by some cell types, but not by others (2, 18). Thus, MYOC is expressed by retinal ganglion cells, photoreceptors, and retinal pigment epithelium, but is not found extracellularly in the retina nor in conditioned medium of retinal pigment cells in culture (2, 18, 19). In contrast, MYOC is found in conditioned medium of trabecular meshwork (TM) cells in culture and in the aqueous humor that bathes the TM in vivo (15, 20–22).

The unique extracellular appearance of MYOC in aqueous humor is interesting because mutations in MYOC cause glaucoma and blindness, but are not associated with any other disease process (23). Glaucoma is a disease commonly associated with elevated intraocular pressure because of decreased outflow of aqueous through the TM tissue (24–29). One possible explanation for the broad expression pattern for MYOC but isolated pathology is that effects of mutant MYOC are tissue-specific. Thus, specificity may be achieved by differences in the normal localization (intracellular versus extracellular) of MYOC and its related function (1, 15–17, 20, 21).

In the present study, we investigate the method of release of MYOC from TM cells in an effort to better understand its function. Our results demonstrate that release of MYOC from TM cells is unlike the mode of release observed for two control proteins that use a conventional secretory mechanism. Moreover, results indicate that MYOC is released from TM cells in association with lipids, from which MYOC dissociates in the medium. We suggest that MYOC is released from TM cells on the outside surface of lipid particles that display biochemical characteristics consistent with exosomes, and which carry an exosome “marker” protein, major histocompatibility complex (MHC) class II (as HLA-DR). Our results also indicate a mechanism by which tissue-specific localization and pathology of MYOC is achieved, and suggests that, in most cells, MYOC is an intracellular protein, but is released with exosomes by the TM.
**Extracellular Myocilin**

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Adenoviral Infection, and Interferon-γ Treatment**

Previously characterized human trabecular meshwork (HTM) cell strains HTM26, HTM29, HTM84, HTM85, HTM86, and HTM90 were isolated and cultured in our laboratory as described (30, 31). Cells (passage 2–4) were grown to confluence and maintained for at least 7 days in low glucose Dulbecco's modified essential medium (DMEM) supplemented with penicillin (100 units/ml), streptomycin (100 mg/ml), glutamine (0.29 mg/ml) solution (Invitrogen), and 10% fetal bovine serum (Hyclone Laboratories, Logan, UT) before experimentation. Prior to addition to culture medium, fetal bovine serum was centrifuged at 141,000 × *g* for 60 min in an SW27 rotor (Beckman Coulter, Fullerton, CA) to remove any particulate material. MCF7 cells (ATCC, Manassas, VA) were cultured as described, except that cell cultures were utilized prior to confluence (at 70–80% confluence) and were never allowed to reach confluence. For experiments examining HLA-DR expression in HTM cell cultures were utilized prior to confluence (at ATCC, Manassas, VA) were cultured as described, except that cell cultures were utilized prior to confluence (70–80% confluence) and were never allowed to reach confluence. For experiments analyzing antibody, incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies in TBS-T containing 5% milk and washed with 0.1 M cacodylate buffer for 1 h. Samples were dehydrated in a graded ethanol series and embedded in Epox 812 resin. Thin sections were cut on a Reichert ultratrac E ultramicrotome, stained with uranyl acetate and lead citrate, and examined on a Philips CM20 transmission electron microscope at 80 kV.

**Isolation of Intracellular Membranes**

Untreated confluent HTM cell cultures were lysed, scraped, and homogenized as described above. Cell lysates were centrifuged first at 1,000 × *g* (PA 45-54-11 rotor) for 1 min to pellet nuclei. Then, supernatants were transferred to and maintained in low serum DMEM (either 1 or 2%) at 4 °C with gentle agitation. Membranes were separated commercially and used at the indicated dilutions: horseradish peroxidase-conjugated anti-tissue plasminogen activator (1:1000, Cedarlane Laboratories Ltd., Ontario, Canada), monoclonal anti-β-actin (1:5000, Sigma), monoclonal anti-CD107a (LAMP1) (1:1000, BD Pharmingen), monoclonal anti-cytochrome oxidase subunit II (1:1000, Molecular Probes), monoclonal anti-GolgI 58K protein (1:1000, Sigma), monoclonal anti-histone deacetylase 2 (1:1000, Sigma), polyclonal rabbit anti-cyclin B (1:1000, Alexis Biochemicals, San Diego, CA), and monoclonal anti-HLA-DR α chain (1:1000, DakoCyto- mation, Carpinteria, CA). All horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson Immunolab- oratories, Inc. (West Grove, PA). For Western blotting, proteins were separated on 10% SDS-PAGE gels, then transferred electrophoretically to nitrocellulose membranes. Membranes were blocked in Tris-buffered saline with 0.2% Tween 20 (TBS-T) containing 5% nonfat dry milk. Primary antibodies were added, and blots were incubated overnight at 4 °C with gentle agitation. Membranes were washed in TBS-T (4 times for 15 min) and, with the exception of the one conjugated primary antibody, incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies in TBS-T containing 5% milk and washed with TBS-T. Proteins were visualized by enhanced chemiluminescence (ECL) (Amer sham Biosciences) on x-ray film (Eastman Kodak Co., Rochester, NY). Where indicated, proteins were quantified in the linear range of the film by densitometry using a bioimaging system and LabWorks4 software (UVP Inc., Upland, CA). For silver staining, proteins were separated on 10% SDS-PAGE gels and visualized using a Bio-Rad silver stain kit as per the manufacturer’s instructions.

**Isolation of Extracellular Membranes**

Extracellular membranes were isolated from the conditioned medium of confluent HTM cells (with or without prior adenosine phosphorylation, or with or without IFNγ treatment) by differential centrifugation as described for the isolation of exosomes (34). Medium was centrifuged first at 10,000 × *g* (SW41 or SW28 rotor) for 60 min to isolate dead cells and cellular debris. Supernatant was transferred to a new tube and centrifuged at 100,000 × *g* for 60 min to isolate extracellular membranes using SW41 or SW28 rotors. Pellets were resuspended in PBS, repelleted (to control for non-membranous protein trapping), and samples were analyzed by matrix-assisted laser desorption ionization qualitative time-of-flight mass spectrometry:mass spectrometry (Proteomics Core Facility, College of Pharmacy, University of Arizona). Protein identity and coverage was determined using SEQUEST software.

**Transmission Electron Microscopy**

Confluent HTM cell monolayers were rinsed with PBS and fixed in 4% paraformaldehyde for 1.5 h, followed by 30 min in 3% glutaraldehyde in 0.1 m cacodylate buffer. Cells were washed 3 times for 10 min each in 0.1 m cacodylate buffer and postfixed in 2% osmium tetroxide in 0.1 m cacodeylate buffer for 1 h. Samples were dehydrated in a graded ethanol series and embedded in Epox 812 resin. Thin sections were cut on a Leica ultracut E ultramicrotome, stained with uranyl acetate and lead citrate, and examined on a Philips CM20 transmission electron microscope at 80 kV.

**Proteomic Analysis**

Two prominent bands observed in silver-stained gels were excised and digested with trypsin. Sequence of protein fragments was identified by matrix-assisted laser desorption ionization qualitative time-of-flight mass spectrometry:mass spectrometry (Proteomics Core Facility, College of Pharmacy, University of Arizona). Protein identity and coverage was determined using SEQUEST software.

**Metabolic Labeling**

Human TM cells were seeded in 6-well culture plates at a density of 1 × 10^6 cells per well. Five to 7 days later, cells were infected as described above. Three to 5 days post-infection, cells in each well were first washed with (3 × 1 ml), then cultured in 1 ml of methionine/
lysates collected at time zero, or were cultured for 4 h in DMEM supplemented at 1 g/ml for the chase period. Medium and conditioned medium was collected. In treated cells, brefeldin A containing a 10 times concentration of Met/Cys, then cells were lysed, conditioned medium was collected. In treated cells, brefeldin A containing a 10 times concentration of Met/Cys, then cells were lysed, conditioned medium was collected. In treated cells, brefeldin A containing a 10 times concentration of Met/Cys, then cells were lysed, conditioned medium was collected. In treated cells, brefeldin A containing a 10 times concentration of Met/Cys, then cells were lysed, conditioned medium was collected. In treated cells, brefeldin A containing a 10 times concentration of Met/Cys, then cells were lysed, conditioned medium was collected. In treated cells, brefeldin A containing a 10 times concentration of Met/Cys, then cells were lysed, conditioned medium was collected. In treated cells, brefeldin A containing a 10 times concentration of Met/Cys, then cells were lysed, conditioned medium was collected. In treated cells, brefeldin A containing a 10 times concentration of Met/Cys, then cells were lysed, conditioned medium was collected. In treated cells, brefeldin A containing a 10 times concentration of Met/Cys, then cells were lysed, conditioned medium was collected. In treated cells, brefeldin A containing a 10 times concentration of Met/Cys, then cells were lysed, conditioned medium was collected. In treated cells, brefeldin A containing a 10 times concentration of Met/Cys, then cells were lysed, conditioned medium was collected.
cles, tPA was used to verify that cytosol was not contaminated with vesicle contents. In contrast to MYOC, tPA was found associated only with intracellular membranes and was excluded from the cytosol. Furthermore, tPA was down-regulated after dexamethasone treatment (Fig. 1A); consistent with a previous report (30).

Whereas the intracellular distribution was similar in the two cell types, MYOC was not detected in conditioned medium of MCF7 cells (Fig. 1C). Interestingly, intracellular MYOC structures in MCF7 cells label in a punctate pattern by immunofluorescence microscopy, similar to intracellular labeling of MYOC in HTM cells (data not shown) (11). Furthermore, the presence of tPA in MCF7 cell culture medium verified that the normal secretory pathway was functional, and confirmed that the extracellular localization of MYOC in HTM cells is different than another cell type expressing native MYOC. Taken together, MYOC in MCF7 cells most likely associates with a population of vesicles that is not shed in the same manner as in HTM cells.

Analysis of Intracellular Membranes—Despite a difference in cytosolic localization for MYOC and tPA, both proteins were observed to sediment with intracellular membranes. Thus we were interested in examining potential differences in intracellular membrane associations. To assess whether MYOC and tPA localized to similar or distinct populations of intracellular membranes, we floated membranes isolated from HTM cell lysates (free of nuclei and large cytoskeletal elements) into linear sucrose gradients (panel B). Antibodies to nuclei, mitochondria, Golgi apparatus, and endoplasmic reticulum markers recognized bands of appropriate sizes in the precleared nuclei fraction but not in the experimental membrane fraction (data not shown). Linearity of gradients was verified by refractometry (inset, panel B). Stable monolayer cell cultures were further examined by transmission electron microscopy (panel C). Shown is an ultrathin section of a human trabecular meshwork cell containing exosome-like vesicles enclosed by multilaminar structures (arrows) (bar = 500 nm). Inset shows magnification of the area outlined by a box to highlight limiting membranes of enclosed vesicles (arrowheads).

![Fig. 2. MYOC-associated membranes in HTM cells equilibrate at a density lighter than traditional secretory vesicles.](image-url)

Cellular membranes were isolated from HTM cell cultures by differential centrifugation and floated into linear sucrose gradients (n = 3). Fractions were collected, and proteins were analyzed by SDS-PAGE/Western blotting (panel A). Protein content of fractions was determined by probing with antibodies specific to MYOC, tPA, lysosomal-associated membrane protein (LAMP1), and cyclophilin B (CyPB). Protein distributions were quantified by densitometry (panel B). Antibodies to nuclei, mitochondria, Golgi apparatus, and endoplasmic reticulum markers recognized bands of appropriate sizes in the precleared nuclei fraction but not in the experimental membrane fraction (data not shown). Linearity of gradients was verified by refractometry (inset, panel B). Stable monolayer cell cultures were further examined by transmission electron microscopy (panel C). Shown is an ultrathin section of a human trabecular meshwork cell containing exosome-like vesicles enclosed by multilaminar structures (arrows) (bar = 500 nm). Inset shows magnification of the area outlined by a box to highlight limiting membranes of enclosed vesicles (arrowheads).
routinely resuspended in PBS and repelleted. To limit trapping and sticking of non-membranous proteins to membranes and to the inside of the centrifuge tube, specific controls were performed; pellets were routinely purified by resuspension in PBS and by repelleting in a clean tube. Furthermore, post-centrifugation medium was collected, and cells were lysed in a hypotonic buffer. Labeled proteins were followed both intracellularly and extracellularly, displaying a uniform distribution of MYOC, as expected from the isolation procedure (Fig. 4A). In MYOC AV-infected cells, recombinant MYOC appeared to accumulate in the extracellular space linearly over time. In cultures infected with β-galactosidase AV, native MYOC expression was not detected at any time point at film exposures tested (Fig. 4A). Because MYOC was detected as early as 6 h in conditioned medium (following media change), recombinant MYOC association with extracellular membranes was evaluated by differential centrifugation (Fig. 4B). Interestingly, the distribution of extracellular, recombinant MYOC was identical to the pattern for native MYOC seen in uninfected culture medium evaluated at 48 h (Fig. 3).

To analyze a potential early association of recombinant MYOC with extracellular membranes, HTM cell cultures were first infected with MYOC AV, and then metabolically labeled with [35S]methionine/cysteine. Conditioned medium was collected at 1 h and subjected to differential centrifugation as before, and analyzed by SDS-PAGE and Western blotting (Fig. 4C). At 1 h, the extracellular distribution of radiolabeled, recombinant MYOC was unlike that seen in previous experiments (Figs. 3 and 4B). Instead, MYOC localized preferentially to debris and extracellular membrane pellets, and appeared in minimal amounts free in the medium, indicating that recombinant MYOC first reaches the extracellular compartment preferentially associated with extracellular membranes.

**Trafficcking of Recombinant MYOC**—Based on our observation of an apparent linear accumulation of recombinant MYOC in the extracellular space, we were interested in addressing whether or not the cellular and extracellular pattern of MYOC displayed properties characteristic of a constitutive secretory protein. To address this question, HTM cell cultures were infected with MYOC AV or with an alkaline phosphatase AV engineered with a signal peptide to facilitate its constitutive secretion (secretory alkaline phosphatase; SeAP), and then metabolically labeled with [35S]methionine/cysteine. Labeled proteins were followed both intracellularly and extracellularly for 6 h. Conditioned medium was collected, and cells were lysed in a hypotonic buffer. Labeled MYOC was immunoprecipitated from lysates or medium with an anti-MYOC IgG, and labeled SeAP was immunoprecipitated with an anti-alkaline phosphatase IgG. Both proteins were analyzed by SDS-PAGE and Western blotting (Fig. 5, A and B). As determined by phosphorimaging, the amount of radiolabeled intracellular MYOC decreased linearly over time, whereas the appearance of labeled MYOC in the extracellular space increased linearly. Interestingly, the mobility pattern of radiolabeled MYOC remained unchanged (both intracellularly and extracellularly), displaying a uniform
doublet throughout the time points evaluated. In contrast, the distribution of SeAP changed dramatically over time, displaying disparity between bands of the doublet intracellularly with a decrease of the lower band and an increase of the upper band, consistent with the existence of a precursor and mature form of the protein. Extracellularly, there was evidence of only the upper broad band, displaying a mobility pattern typical of a traditional secretory protein. The observation that the mobility pattern of the MYOC doublet remained unaltered further suggests it is not post-translation-
ally modified in the ER-Golgi and is not processed as a typical secretory protein.

Effect of Blocking ER-Golgi Transport on MYOC Trafficking—The apparent dissimilarities between the processing of recombinant MYOC and SeAP led us to question whether, in HTM cells, these proteins are both similarly trafficked through the endoplasmic reticulum and Golgi apparatus. As before, we infected HTM cell cultures with either a MYOC or SeAP AV, metabolically labeled the cultures with [35S]methionine/cysteine, and this time either treated the cells with 1 μg/ml brefeldin A or left the cells untreated. Labeled proteins were followed for 4 h, at which time cell lysates and medium were collected, and MYOC and SeAP proteins were isolated by immunoprecipitation as before. Equivalent samples were analyzed by SDS-PAGE and Western blot (Fig. 6, A and B). Treatment with brefeldin A halted secretion of both MYOC and SeAP at 4 h. Interestingly, the mobility pattern of the MYOC doublet remained unchanged between treated and untreated cells, suggesting that MYOC does not undergo post-translational modification in the ER-Golgi. Contrastingly, in SeAP AV-infected cells, treatment with brefeldin A completely eliminated the upper broad band, but not the lower band of the doublet, suggesting that trafficking through the Golgi and post-translational modification is required to produce the mature form of the protein.

Buoyant Properties of Extracellular Membranes—The association of MYOC with an extracellular membrane fraction led us to next question whether this fraction displayed biochemical properties similar to those described for exosomes (41, 42). Despite the absence of a single marker protein found consistently in every exosome preparation from different cell types, we wanted to determine whether exosome preparations from HTM cells contain MHC class II antigens, the most common protein class found in exosome preparations (42, supplemental Table S1). MHC class II antigens are proteins involved in antigen recognition in the immune system. As the TM has been implicated to have an immune function and has been shown to express the MHC class II antigen, HLA-DR, in vivo (43, 44), we sought to determine whether HLA-DR was present in extracellular membranes from HTM cells. HTM cells cultured in vitro require stimulation with IFNγ to induce expression of HLA-DR (43, 44), consistent with other cell types that shed exosomes containing MHC class II antigens (38, 45). Hence, we either treated confluent HTM cell cultures with 500 units/ml of human IFNγ for a period of at least 5 days or left the cells untreated. At 48 h after medium change, conditioned medium and cell lysates were collected, and medium was processed by differential centrifugation as before. Samples of cell lysate and extracellular membranes from treated and untreated cells were analyzed by SDS-PAGE and Western blot, and the expression of HLA-DR and MYOC was analyzed using specific antibodies (Fig. 7A). As expected, both treated and untreated cells expressed similar levels of MYOC in both cell lysate and extracellular membrane fractions; yet only IFNγ-treated cells expressed HLA-DR. Significantly, we observed that extracellular membranes from IFNγ-treated cells contained the MHC class II antigen (Fig. 7A, lane 4), consistent with reports of exosomes from other cell types (34, 37–40, 45–51).

To test whether MYOC and HLA-DR are found associated with extracellular membrane populations of similar densities, sucrose gradients were layered on top of purified preparations of pooled extracellular membranes from IFNγ-treated and untreated cells (essentially as previously described for the analysis of exosomes). Gradients were ultracentrifuged overnight, and fractions were collected and analyzed for the presence of MYOC and HLA-DR. Fractions from untreated cells were also analyzed by SDS-PAGE followed by silver staining for the distribution of all membrane-associated proteins. Silver staining revealed that the majority of proteins equilibrated at a density of 1.142 g/ml (Fig. 7B). Western blotting showed that MYOC equilibrated preferentially with these proteins at a density peak of 1.142 g/ml (Fig. 7B), whereas HLA-DR was undetectable in untreated cells (data not shown). In contrast, in IFNγ-treated cells, HLA-DR (Fig. 7C) and MYOC (data not shown) equilibrated in the same fractions, having a density of 1.128–1.147 g/ml, consistent with the reported density of exosomes in other cell types (34, 37, 46).

Degradation Analysis by Proteinase K—To assess the orientation of endogenous MYOC on extracellular membranes, conditioned medium was subjected to sequential differential centrifugation as before, except that prior to high-speed ultracentrifugation, medium was divided equally in half. Purified pellets were incubated in a protease K or a control buffer. Equivalent fractions were analyzed for the presence of MYOC (Fig. 8). Analyses indicated that MYOC was completely degraded in the presence of proteinase K, and was not degraded by a control buffer under identical conditions. This finding indicates that endogenous MYOC is localized to the outside of the extracellular membranes.
DISCUSSION

In this study, we are first to follow nascent MYOC over time and show that MYOC, in a cell-type specific manner, exits the cell via a mechanism atypical of secretory proteins. We show that, in two different cell types, native MYOC localizes to the cytosol and intracellular membranes, suggesting that MYOC is an intracellular protein with the capacity to associate with and function in the secretory pathway. Such a distribution differed from a secreted protein, tPA, which was excluded from the cytosol and found limited to cellular fractions containing membranes. Interestingly, in both cell types tested, tPA was located extracellularly as expected; however, MYOC was only found outside of one of the cell types: HTM cells.

To examine more closely the unique extracellular appearance of MYOC in HTM cells, we utilized an adenovirus expression system in combination with a metabolic labeling protocol. We followed MYOC over time and compared the processing of nascent MYOC in HTM cells to a secreted protein, SeAP. For example, we show that the characteristic mobility pattern of MYOC does not change between intracellular and extracellular compartments over time. Our observations of a uniform extracellular doublet at 1 h, plus the similarity between mobility patterns at 0 and 1 h in cell lysates, are consistent with the idea that insufficient time has elapsed for MYOC to translocate through the ER and Golgi and undergo post-translational modification. In fact, blocking ER-Golgi transport inhibits glycosylation of SeAP controls, but has no effect on the mobility pattern of the MYOC doublet, supporting the argument that MYOC does not undergo Golgi-mediated post-translational modification. This finding is consistent with a previous study (52) and in opposition to another (53) that examined changes in the mobility pattern of MYOC following treatment with enzymes that cleave sugar groups.

Except for excluding glycosylation, the scope of the present study did not include determining experimentally the nature of the two forms of MYOC on SDS-PAGE. We speculate that the doublet may be a consequence of one of a number of possibilities: first, MYOC may be subject to another type of post-translational modification, such as phosphorylation; second, MYOC may utilize two alternate start sites (Met-1 and Met-15); third, MYOC may have two protein conformations that migrate differently on SDS-PAGE. Further research is necessary to determine the exact nature of the MYOC doublet.

Evidence of an alternative mechanism of release of MYOC from HTM cells was demonstrated in experiments showing that both native and recombinant MYOC co-purify with extracellular membranes having biochemical properties of exosomes. Our temporal characterization of extracellular MYOC illustrates that, at early time points after release, the majority of MYOC was found associated with exosome-like membranes. But, by 48 h, most of MYOC was a free protein, suggesting that MYOC was released from HTM cells bound to membranes from which it dissociates over time into the medium. Consistent with this idea, MYOC in the extracellular, exosome-like fraction from HTM cells is susceptible to protease digestion. MYOC likely associates with the secretory pathway in a manner not requiring a functional signal sequence. Consistent with our data showing a cytosolic localization of endogenous MYOC and a mobility pattern unaffected by brefeldin A, others have shown that the putative signal sequence of MYOC is
non-functional in HTM cells (1) and transfected cells (54). In contrast, in a cell-free system, the putative signal peptide of MYOC was either functional or nascent MYOC bound tightly with reticulocyte membranes (55). We suggest that the signal peptide is inactive and that the N-terminal coiled-coil of MYOC facilitates interactions with the secretory pathway, much like that of proteins of the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) family. Thus, on the surface of exosomes, MYOC may function in the secretory pathway that is responsible for exosome targeting or release into the extracellular compartment. Because MYOC is a cytosolic protein that also associates with cellular membranes, we propose that it may interact via its coiled-coil with the complimentary coiled-coil of a transmembrane protein located on the exosomal surface, as part of a cytoplasmic coat. The deficiency of extracellular MYOC in other cell types may be explained by the association of MYOC with a different population of intracellular vesicles that are not released as exosomes or may be related to a lower (or lack of) production and release of exosomes in other MYOC-expressing cell types. Additional work is needed to resolve these possibilities.

The association of MYOC with extracellular membranes containing HLA-DR, an MHC class II antigen, that display exosome-like properties is the first evidence to place MYOC in a specific cellular pathway. Interestingly, no single protein or group of proteins was found consistently in exosomes from different cell types. Most exosome-associated proteins appear to be cell type-specific. Hence, exosomes are typically classified not by their protein composition but by biochemical properties, as was done in the present study. However, in two-thirds of the cell types described to release exosomes, MHC class II is a prominent component of these vesicles (34, 37–40, 45–51). Our observation that MYOC colocalizes with HLA-DR on membranes with a density typical of exosomes is compelling evidence that the release of MYOC from HTM cells occurs via an exosome-mediated mechanism. Exosomes have been described in a number of cell types including B lymphocytes, reticulocytes, dendritic cells, and intestinal epithelium (34, 39, 40, 56). In such cells, exosomes have been proposed to function in immune response processes and intercellular communication (57). Because the TM is both in direct contact with the aqueous humor that passes through the anterior chamber of the eye and positioned at a strategic location between the anterior chamber and the venous circulation (the blood-aqueous barrier), and because there is a lack of lymphatic vessels draining the intraocular compartment, it is likely that the uptake and processing of antigens may occur in the TM prior to reaching the venous blood circulation (44). An attractive possibility based on the physiology and architecture of the TM is that MYOC-associated exosomes function in the initiation of ocular immune responses that may play a role in the regulation of intraocular pressure in the normal and glaucomatous human eye. The connection between mutations in MYOC, immune function, and glaucoma is presently unknown.

Understanding the function of MYOC was of clinical importance. Mutations in MYOC link only to forms of open angle glaucoma, despite a near ubiquitous tissue expression pattern (2, 14, 23). Thus, the function of MYOC is likely crucial only in the eye. Given that all patients with MYOC-linked glaucoma have elevated intraocular pressure, MYOC likely has a vital function at the primary site of intraocular pressure regulation: the trabecular meshwork. Our results suggest that MYOC is released from HTM cells, but not other cells as previously reported. Furthermore, we suggest that HTM cells traffic MYOC in a unique fashion, and specifically release MYOC coincident with exosomes. Thus, we hypothesize that the cell type-specific release of MYOC by HTM cells may account for the tissue-specific nature of the pathology caused by MYOC mutations.

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REFERENCES

1. Nguyen, T. D., Chen, P., Huang, W. D., Chen, H., Johnson, D., and Polansky, J. R. (1998) J. Biol. Chem. 273, 6341–6350
2. Kuba, R., Noda, S., Wang, Y., Minoshima, S., Asakawa, S., Kudoh, J., Mashima, Y., Oguchi, Y., and Shimizu, N. (1997) Genomics 34, 360–369
3. Kubota, K., Yamanoto, T., Yasita, K., Danielson, P., Kobayashi, H., Ohshiro, K., Funaki, H., Koyama, Y., Fujinaka, H., Kawasaki, K., Sutcliffe, J., Arakawa, M., and Kihara, I. (2000) J. Am. Soc. Neur. 11, 803–813
4. Bal, R., and Anholt, R. (1995) Biochemistry 32, 1047–1053
5. Danielsson, P., Fors-Petter, S., Battenden, E., deLere, L., Bloom, F., and Sutcliffe, J. (1994) J. Neurosci. Res. 38, 468–478
6. Karavanich, C., and Anholt, R. (1998) Mol. Biol. Ecol. 15, 718–726
7. Nagano, T., Nakamura, A., Mori, Y., Maeda, M., Takami, T., Shinoda, K., Takagi, H., and Sato, M. (1998) Mol. Brain Res. 53, 13–23
8. Snyder, D., Rivers, A., Yokoe, H., Mence, B., and Anholt, R. (1991) Biochemistry 30, 9143–9155
9. Leblonova, V., Davetov, B., Sterling, A., Rahman, M., Grishin, E., Totty, N., and Ushkaryov, Y. (1997) J. Biol. Chem. 272, 26715–26724
10. Sugita, S., Ichihnenko, K., Khotrochv, M., and Sudhof, T. (1998) J. Biol. Chem. 273, 27115–27124
11. Stammer, W. D., Roberts, B. C., Howell, D. N., and Epstein, D. L. (1998) Invest. Ophthalmol. Vis. Scis. 39, 1804–1812
12. Ortega, J., Eckhard, J., and Coca-Prados, M. (1997) FEBS Lett. 413, 349–353
13. Fingert, J. H., Ying, L., Swiderski, R. E., Nystuen, A. M., Arbour, N. C., Alward, W. L., Shefield, V. C., and Stone, E. M. (1998) Genome Res. 8, 377–384
14. Adam, M. F., Belmont, A., Biniati, P., Brezin, A. P., Valtot, F., Bechette, A., Dascotte, J. C., Copin, B., Gomez, L., Chaventre, A., Bach, J. F., and Garrod, H. J. (1997) Hum. Mol. Genet. 6, 2091–2097
15. Ueda, J., Wentz-Hunter, K. K., Cheng, E. L., Fukuchi, T., Abe, H., and Yue, B. Y. T. (2000) J. Histochem. Cytochem. 48, 1321–1329
16. Sohn, S., Hur, W., Joe, M. K., Kim, J. H., Lee, Z. W., Ha, K. S., and Kee, C. (2000) Invest. Ophthalmol. Vis. Scis. 41, 3680–3685
17. Wentz-Hunter, K., Ueda, J., Shimizu, N., and Yue, B. Y. T. (2002) J. Cell. Physiol. 196, 46–53
18. Polansky, J. R., Faus, D. J., Chen, P., Chen, H., Lutjen-Drecoll, E., Johnson, D., Kurtz, R. M., Ma, Z. D., Bloom, F., and Nguyen, T. D. (1997) J. Ophthalmol. 211, 126–139
19. Tamm, E. R., Russell, P., Epstein, D. J., Johnson, D. H., and Piagotorsky, J. (1999) Invest. Ophthalmol. Vis. Scis. 40, 2577–2582
Extracellular Myocilin

20. Filla, M. S., Liu, X., Nguyen, T. D., Polansky, J. R., Brandt, C. R., Kaufman, P. L., and Peters, D. M. (2002) Invest. Ophthalmol. Vis. Sci. 43, 151–161
21. Ohmann, A., Goldwich, A., Flugel-Koch, C., Fuchs, A., Schwager, K., and Tamm, E. (2003) Glia 43, 128–140
22. Rao, P. V., Alingham, R. R., and Epstein, D. L. (2000) Exp. Eye Res. 71, 637–641
23. Stone, E. M., Fingert, J. H., Alward, W. L. M., Nguyen, T. D., Polansky, J. R., Sunden, S. L. F., Nishimura, D., Clark, A. F., Nystuen, A., Nichols, B. E., Mackey, D. A., Ritch, R., Kalenak, J. W., Craven, E. R., and Sheffield, V. C. (1997) Science 275, 668–670
24. Rohen, J. (1983) Invest. Ophthalmol. 22, 183, 1161–1172
25. Wiederhold, M. (1998) Curr. Opin. Ophthalmol. 9, 66–69
26. Lutjen-Drecoll, E. (1998) Prog. Retinal Eye Res. 17, 91–119
27. Johnstone, R. M., Adam, M., Hammond, J. R., Orr, L., and Turbide, C. (1987) J. Cell Biol. 105, 262–271
28. Wubbolts, R., Leckie, R. S., Veenvliet, P., Schwarzmann, G., Mobius, W., Hoershemeyer, J., Slot, J. W., Geuze, H. J., and Stoorvogel, W. (2003) J. Biol. Chem. 278, 10963–10972
29. Clayton, A., Court, J., Navabi, H., Adams, M., Mason, D. M., Hobet, J. A., Newman, G. R., and Jasani, B. (2001) J. Immunol. Method 247, 163–174
30. Tripathi, B. J., Tripathi, R. C., Wong, P., and Raja, S. (1990) Invest. Ophthalmol. Vis. Sci. 1903–1907
31. Tripathi, B. J., Tripathi, R. C., Wong, P., and Raja, S. (1990) Invest. Ophthalmol. Vis. Sci. 14, 20121–20127
32. Tripathi, B. J., Tripathi, R. C., Wong, P., and Raja, S. (1990) Invest. Ophthalmol. Vis. Sci. 14, 611–617
33. Tripathi, B. J., Tripathi, R. C., Wong, P., and Raja, S. (1990) Invest. Ophthalmol. Vis. Sci. 14, 594–600
34. Tripathi, B. J., Tripathi, R. C., Wong, P., and Raja, S. (1990) Invest. Ophthalmol. Vis. Sci. 14, 584–593
35. Tripathi, B. J., Tripathi, R. C., Wong, P., and Raja, S. (1990) Invest. Ophthalmol. Vis. Sci. 14, 575–583
36. Tripathi, B. J., Tripathi, R. C., Wong, P., and Raja, S. (1990) Invest. Ophthalmol. Vis. Sci. 14, 566–574
37. Tripathi, B. J., Tripathi, R. C., Wong, P., and Raja, S. (1990) Invest. Ophthalmol. Vis. Sci. 14, 556–565
38. Tripathi, B. J., Tripathi, R. C., Wong, P., and Raja, S. (1990) Invest. Ophthalmol. Vis. Sci. 14, 546–555
39. Tripathi, B. J., Tripathi, R. C., Wong, P., and Raja, S. (1990) Invest. Ophthalmol. Vis. Sci. 14, 536–545
40. Tripathi, B. J., Tripathi, R. C., Wong, P., and Raja, S. (1990) Invest. Ophthalmol. Vis. Sci. 14, 526–535
41. Tripathi, B. J., Tripathi, R. C., Wong, P., and Raja, S. (1990) Invest. Ophthalmol. Vis. Sci. 14, 516–525