Localization of Myocilin to the Golgi Apparatus in Schlemm’s Canal Cells

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PURPOSE. Biochemical and genetic evidence suggests that overexpression of or mutations in myocilin within the cells of the aqueous humor outflow pathway play a significant role in the development of steroid-induced and several other open-angle glaucomas. As a baseline to understanding the normal and pathologic function of myocilin, we determined the subcellular localization of myocilin in steroid-treated human Schlemm’s canal endothelial (SC) cells.

METHODS. SC cells were grown to confluence, treated with dexamethasone for 10 days, and then stained using antibodies against myocilin, tubulin, or β-COP (a specific golgi protein) or vital stains for endoplasmic reticulum (ER) and golgi. Brefeldin A (BFA) and nocodazol (NZ) were used to disrupt the golgi or microtubules.

RESULTS. The authors found that myocilin staining was (a) always centered around the centrosome, (b) very similar to the pattern seen with NBD-ceramide, (c) was disrupted in characteristic ways by BFA and NZ and (d) showed extensive colocalization with β-COP.

CONCLUSIONS. Results indicate that myocilin is localized to the golgi in SC cells. Such localization is consistent with myocilin being processed for secretion but is also consistent with sequence analysis and other data that suggest that myocilin or myocilin mutations might be targeted to the cytoplasmic face of the golgi, and under some circumstances play a role in or interfere with golgi or vesicle function. How such interference could eventually lead to open angle glaucoma is discussed. (Invest Ophthalmol Vis Sci. 2000;41:3842–3849)

The trabecular meshwork-inducible glucocorticoid response/myosin-ciliary stalk-related (TIGR/myocilin) protein has been implicated by biochemical and genetic evidence to be in the pathway that results in either steroid-induced or several additional open-angle glaucomas.1–11 Myocilin was initially of interest to glaucoma research because trabecular meshwork (TM) endothelial cells greatly increased their expression of the protein in response to treatment with glucocorticoids. Thus, myocilin provided a mechanistic link to the area in chromosome 1 that coded for myocilin.3 Associated with juvenile onset open angle glaucoma were studies to identify genes associated with diseases of the retina that myocilin positively regulated.12

Tubulin, a peripheral protein, had named the protein myocilin because of sequence homology to myosin in the N-terminal half of the protein and because of its localization to the ciliary root of the rod inner segment.12 Interest in myocilin increased dramatically when mutations in myocilin were linked to the area in chromosome 1 that coded for myocilin.4 Many subsequent genetic linkage studies have demonstrated a strong relationship between mutations in myocilin, specifically in the C-terminal, olfactomedin-homology domain, with both juvenile and adult onset open angle glaucomas.1,4–11,13 Yet the role that myocilin normally plays in outflow pathway cells and how mutated forms of myocilin might cause reduced outflow, increased intraocular pressure, and loss of vision have not been established.

Using antibodies generated in the laboratory of Jon Polansky to full-length recombinant myocilin,5 we have recently shown that human Schlemm’s canal endothelial (SC) cells also express myocilin in culture when exposed to dexamethasone (Dex) but that the pattern of staining is different from that seen in TM cells.14 Steroid-treated TM cells exhibited myocilin staining throughout the cell body, whereas in SC cells myocilin staining was confined to a ribbon-like compartment near the nucleus. Because the extensive staining for myocilin in TM cells, and more so in nonsteroid controls, would have overwhelmed particular localizations, we used SC cells to determine the identity of the myocilin-stained organelle. Our hypothesis was that myocilin-stained organelle was either the golgi apparatus and small vesicles or the ER. Identification of the particular compartment found to contain myocilin staining is a useful first step in understanding the functional role myocilin plays in the cells of the outflow pathway.

METHODS

SC cells were obtained from nonglaucomatous human donor tissue using methods described previously.1–3 SC cell cultures of characteristic growth rate and fusiform shape at confluence were grown in Medium 199 (Gibco, Grand Island, NY) with 12% FBS, penicillin, streptomycin, and amphotericin B, at 36°C in 5% CO2. For experiments, cultures of less than passage 8 were plated onto gelatin-coated glass coverslips in six-well dishes.

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plates and grown to confluence. At confluence, the serum concentration was reduced to 2%, and the cells were treated with 1 μM Dex (Sigma, St. Louis, MO) in EtOH, or EtOH control, such that EtOH was 0.1% by volume. The medium was replaced on the fifth day after Dex addition, and cells were maintained for 10 days total in the presence of Dex. After 10 days in Dex, the cells were washed briefly in PBS and fixed in 4% freshly made formaldehyde in PBS for 5 minutes, permeabilized for 5 minutes with 0.5% Triton X-100 in PBS, and washed three times in PBS with 5 minutes between washes. Primary antibodies to myocilin (anti–full-length recombinant myocilin polyclonal, kindly provided by Jon Polansky), β-COP (Sigma, clone maD), β-tubulin (Sigma, clone Tub 2.1), acetylated tubulin (Sigma, clone 6-11B-1), and nonimmune rabbit or

Figure 1. Pattern of myocilin staining in SC cells relative to the primary cilium. Immunofluorescence micrographs of acetylated tubulin (A, C, E, G) and myocilin (green), acetylated tubulin (red) and DAPI (blue) (B, D, F, H). Each left-right pair shows the same view. Arrows point to the primary cilium of each cell. Scale bar, 10 μm.
FIGURE 2. Comparison of endoplasmic reticulum versus golgi stains in live SC cells using vital stains for ER (A, B) and golgi (C, D). Original magnification, ×25 (A, C), ×100 (B, D). Scale bars, 50 and 10 μm, respectively.

FIGURE 3. Myocilin staining after nocodazol treatment. Immunofluorescence micrographs of SC cell in the absence (A) or presence (B) of 10 μM nocodazol for 30 minutes. Myocilin is green and β-tubulin stained microtubules are shown in red (rhodamine). Scale bar, 10 μm.

FIGURE 4. Effect of BFA on golgi structure. SC cells were treated with vehicle control (A, C) or 5 μg/ml BFA (B, D). For live cell studies (A, B), 15 minutes after addition of BFA or control the cells were stained with NBD C6 ceramide and observed at 37°C. Parallel control (C) or BFA-treated (D) cells were fixed after 30 minutes in BFA and stained for myocilin. Original magnification, ×25 (A, B; scale bar, 50 μm) or ×100 (C, D; scale bar, 10 μm).
mouse serum were used, at the appropriate dilutions. After washing, goat anti-rabbit FITC and anti-mouse TRITC-conjugated secondary antibodies (affinity purified IgGs; BioSource, Camarillo, CA) and 1/1000 dilution of diamidino phenylindole (DAPI; Sigma) were used to stain the primary antibodies and DNA.

For live cell experiments, no DEX exposure was used. Vital stains specific for the ER and golgi (ER tracker or NBD C6-ceramide; Molecular Probes, Eugene, OR) were added to the cells for 20 to 30 minutes at 36°C, and then the coverslips were removed and quickly placed face down on a cleaned glass microscope slide. Two layers of double-sticky cellophane tape were used to provide a space between the coverslip and slide. Medium without stain was added to the space, and the chamber was then sealed with fingernail polish. The cells were then placed in a warm (35–36°C) chamber surrounding a Zeiss Axioplan (Thornwood, NY) upright microscope and viewed with appropriate filter sets, and 25× and 100× plane fluor objectives.

For drug addition experiments with cells that would be viewed live or fixed, brefeldin A (BFA; Sigma) was used at a final concentration of 5 μg/ml. Fifteen microliters of a 1 mg/ml stock was added to 3 ml of medium. Nocodazole (Sigma) was dissolved in DMSO at 10 mM stock, and added as a 1/1000 dilution to the cells (3 μl/3 ml). Controls had vehicle (DMSO or PBS) added at the same time and volume. Drugs were present for 30 minutes before fixation or viewing live cells. For live cell experiments, the vital stain was added 10 minutes before drug addition. All experiments were replicated three or more times with three independent SC lines (from normal donors). Control cells in the same figure are from the same experiment with cells treated with the appropriate vehicle(s). Images for both fixed and live cells were obtained using a Photometrics CH250 cooled CCD camera using Image Processing Laboratory (Scanalytics, Inc., Billerica, MA) software. Only Figure 3 used a sharpening filter (unsharp mask; 250%, 3.2 pixel radius and two-level threshold) within Photoshop (Adobe, Inc., San Jose, CA) and only on the red channel. Color figures were merged in the IP laboratory from individual grayscale images.

**Results**

To ascertain the position of myocilin staining within the cell relative to the centrosome, we doubly stained Dex-treated SC cells with antibodies to both myocilin and to acetylated tubulin. We tried several antibodies that we expected to stain the cells with antibodies to both myocilin and to acetylated tubulin antibodies. However, we had noticed that SC cells, being endothelial, consistently showed a prominent primary cilium that stained with either β-tubulin or acetylated tubulin antibodies. Figure 1 shows the pattern of myocilin staining relative to the primary cilium. The left column (A, C, E, G) shows the rhodamine channel only, with the primary cilium identified with arrows. The cilia were each 4 to 6 μm long and always situated adjacent to one “end” of the each elongated nucleus. Figures 1B, 1D, 1F, and 1H show the same cells as on the left, but with both the FITC channel and DAPI channel included. Note that myocilin typically stains only one cell per view, consistent with our earlier results that only 5% to 15% of cells stain for myocilin even after Dex treatment. However, every cell that had myocilin staining showed the myocilin surrounding that cell’s primary cilium. This is what would be expected if myocilin was present in or attached to the surface of the golgi apparatus.

Figure 2 compares vital stains for ER and golgi using ER tracker (Figs. 2A, 2B) and NBD C6-ceramide (Figs. 2C, 2D). We present ER tracker as a grayscale image because when presented in blue, it did not show sufficient detail. The left panels (Figs. 2A, 2C) show lower magnification (25× objective) views of living SC cells, whereas the panels on the right show 100× views (scale bars, 50 and 10 μm, respectively). ER tracker shows very fine tubelike stings throughout the cells. These are only barely visible at low magnification but are readily apparent in 100× views. In contrast, the NBD-ceramide stain shows a relatively compact, ribbonlike compartment near the nucleus in every cell, as seen with myocilin.

If myocilin colocalizes with the golgi, it should be dependent on intact microtubules for its localization to the peri-centrosomal region. We therefore asked whether myocilin localization would be significantly altered by nocodazole (NZ), which rapidly disassembles all but the most stable cellular microtubules. As a final test of the specificity of myocilin to the golgi, we show the myocilin staining pattern at high magnification. In contrast to the NZ results, BFA consistently caused a much less punctate, more diffuse staining pattern with myocilin than the large vesicular staining pattern of myocilin seen after NZ, consistent with dispersal of the golgi membrane into the ER. Note that in Figure 4C and to a lesser extent in 3A, the myocilin-stained compartment extended further around the nucleus than usual. However, this is consistent with ceramide staining, which shows that although the majority of staining is confined to one side of the nucleus, sometimes a small projection of golgi membrane extends part way around the nucleus.

As a final test of the specificity of myocilin to the golgi, we stained cells with antibodies to both myocilin and to the golgi-specific protein β-COP. Figure 5 shows a comparison between myocilin (A, D, G, J) and β-COP (B, E, H, K) localization. Figures 5C, 5F, 5I, and 5L show the merged image of FITC and rhodamine fluorescence. The cells in the last two rows (G through L) had been treated with NZ (G through J) or BFA (J through L) as described above. Although myocilin and β-COP appeared to stain identical compartments, the myocilin stain consistently appeared more punctate than did β-COP. After treatment with NZ, myocilin
staining moved throughout the cell body in many distinct vesicle-like components (Figs. 5G and 5I and Fig. 3B). β-COP did not show the same dispersed, bright vesicular staining pattern after NZ, but rather a golgi remnant was visible in most cells at the time point tested (30 minutes) along with a diffuse background staining (Fig. 5H). Both β-COP and myocilin dispersed after BFA, consistent with both dispersing into the ER, but the regions of maximal staining sometimes did not coincide, as shown in Figure 5L.

**DISCUSSION**

Our results are consistent with localization of myocilin to the golgi apparatus. Identification of the myocilin-stained organelle as the golgi is based on (1) the location of staining centered around the microtubule organizing center (centrosome); (2) strong similarity between the pattern of myocilin staining and that seen with NBD-ceramide; (3) dispersal of myocilin staining with BFA and NZ in a manner almost identical with that seen...
TABLE 1. Golgi and Microtubule Motor Proteins with Homology to Myocilin

<table>
<thead>
<tr>
<th>Protein</th>
<th>Accession No.</th>
<th>% Amino Acids Identical or Conserved*</th>
<th>Sequence Showing Homology†</th>
<th>No. of Proteins with Greater Homology‡</th>
<th>Expected Value§</th>
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<tr>
<td>88kDa golgi protein</td>
<td>AF204231</td>
<td>23.4</td>
<td>43–367</td>
<td>6</td>
<td>6e–40</td>
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<tr>
<td>Golgi-associated microtubule-binding protein</td>
<td>Y12490</td>
<td>25.4</td>
<td>35–243</td>
<td>8</td>
<td>5e–56</td>
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<tr>
<td>Golgin subfamily a and b</td>
<td>NM_004487, NM 008146.1</td>
<td>21.4</td>
<td>48–229</td>
<td>17</td>
<td>1e–19</td>
</tr>
<tr>
<td>36kD golgi complex-protein</td>
<td>JC5837</td>
<td>24.4</td>
<td>32–238</td>
<td>23</td>
<td>4e–15</td>
</tr>
<tr>
<td>Golgin 67</td>
<td>AF163441</td>
<td>10.7</td>
<td>75–231</td>
<td>28</td>
<td>5e–14</td>
</tr>
<tr>
<td>Trans-Golgi p230</td>
<td>U41740</td>
<td>18.6</td>
<td>34–230</td>
<td>52</td>
<td>1e–11</td>
</tr>
<tr>
<td>256 kD golgin protein</td>
<td>CA58041</td>
<td>16.7</td>
<td>45–226</td>
<td>52</td>
<td>1e–11</td>
</tr>
<tr>
<td>Golgin-245</td>
<td>AAC51791.1</td>
<td>16.7</td>
<td>45–226</td>
<td>52</td>
<td>7e–11</td>
</tr>
<tr>
<td>Golgin-160</td>
<td>P55937</td>
<td>23.4</td>
<td>34–230</td>
<td>72</td>
<td>3e–9</td>
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<tr>
<td>Dynactin (human, mouse, chick, rat)</td>
<td>Q14203, QCAA44617, P35458, P28023</td>
<td>20.2</td>
<td>29–224</td>
<td>9</td>
<td>7e–50, 1e–30, 2e–32, 8e–51</td>
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<tr>
<td>Mitotic kinesin-like protein</td>
<td>AAF00594.1</td>
<td>21.6</td>
<td>51–225</td>
<td>10</td>
<td>2e–22</td>
</tr>
</tbody>
</table>

* % homology was calculated as the number of identical amino acids plus the number of conserved substitutions in the matched sequence divided by the 504 amino acids in full length myocilin.
† Based on the 504 aa of myocilin, N-to-C terminal.
‡ Only distinct, non-hypothetical, non-golgi related eucaryotic proteins have been included. Myocilin itself was not included, and all olfactomedins and myocins were counted as one each.
§ Expected value is the chance that the amino acid sequence correspondence occurred by chance. Lower numbers (higher negative numbers) imply greater significance. The reported values were obtained after 1 iteration using PSI–BLAST software.
\[This is a subset of the 11 dynactin isoforms picked up by PSI–BLAST.\]
obtained. Direct inspection of the human myocilin sequence disclosed three additional motifs homologous to "golgi localization domains," and all three were in the olfactomedin domain. These sequences were located at amino acid numbers 298 (FEYDL), 369 (FPYS), and 451 (FAYD). Each of the sequences contains a tyrosine two-amino acid C-terminal to a phenylalanine, which is thought to be most critical to golgi targeting. Although further work will be necessary to determine if any of the identified sequences confer golgi localization, it should be noted that these sequences would act along with the motifs found in the N-terminal portions of myocilin and that even low affinity may be all that is necessary to produce localization.

Recent evidence has shown that open angle glaucoma-related mutations in myocilin result in the protein becoming part of a triton-insoluble pool, and being blocked from secrete. Thus, it becomes likely that myocilin mutations cause pathology by acting intracellularly. A mechanism to explain pathogenesis by intracellular, mutated myocilin would be if altered myocilin was targeted to the cytoplasmic face of the golgi and vesicles and now blocked the function of normal golgi and/or motor proteins, thus interfering with golgi trafficking, sorting, and transport functions. Such interference would vary in extent and kind with the type of mutation present, but even a relatively slight interference in golgi function would likely decrease cell viability. Such a mechanism could be a factor in the best-documented evidence of cellular pathology of the outflow pathway, the decreased number of TM cells on the trabeculae in patients with primary open angle glaucoma. Such loss of cellularity would expose the underlying collagen and extracellular matrix to enzymatic degradation and collapse, closing pathways for aqueous outflow and gradually raising intraocular pressure. Thus, although SC cells have proven useful in proving myocilin localization to the golgi, future studies will return to TM cells to assess the effects of myocilin mutations on cellular viability, intracellular myocilin localization and movement, and golgi vesicle function.

Acknowledgments

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References