Dense Nuclear Cataract Caused by the γB-Crystallin S11R Point Mutation

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PURPOSE. To identify the causative gene mutation for a new dominant cataract in mice and to investigate the molecular basis for how the mutated gene leads to a dense nuclear cataract.

METHODS. Genomewide linkage analysis and DNA sequencing were used to determine the gene mutation. Histology, immunohistochemistry, and Western blotting were used to characterize lens phenotypes. Ion concentrations were measured by an inductively coupled plasma–optical emission spectrometer (ICP-OES).

RESULTS. A point mutation (A to C) of the γB-crystallin gene, which results in the γB-S11R mutant protein, was identified in this cataractous mouse line. Homozygous mutant mice developed dense nuclear cataracts associated with disrupted inner lens fiber cells. Immunohistochemistry data revealed γ-crystallin aggregates at the cell boundaries of inner mature fibers that lose actin filaments. Western blotting showed an increased degradation of crystallin proteins correlated with the nuclear cataract. ICP-OES confirmed a substantial elevation of calcium concentration in mutant lenses.

CONCLUSIONS. This dominant cataract was caused by the γB-S11R mutation. Mutant γB-S11R proteins triggered the γ-crystallin aggregation that probably disrupted membrane-cytoskeleton structures of inner fiber cells, causing increased calcium influxes. Subsequent activation of calcium-dependent protein degradation and degeneration of inner mature fiber cells led to the dense nuclear cataract. (Invest Ophthalmol Vis Sci. 2008; 49:304–309) DOI:10.1167/iovs.07-0942

The eye lens is composed of lens cells wrapped by a collagen-based membrane, known as the lens capsule. Lens cells are predominantly elongated fiber cells covered by a monolayer of epithelial cells at the surface of the anterior hemisphere.1 Lens inner fiber cells are mature fibers that lack intracellular organelles to minimize light scattering. Thus, only anterior surface epithelial cells and newly differentiated fiber cells in the lens periphery contain intracellular organelles, such as endoplasmic reticulum, Golgi, and mitochondria; those cells are able to synthesize proteins or metabolites that maintain the homeostasis of organelle-free inner fiber cells. Several studies have begun to unravel some insights about fiber cell maturation.2–4

Crystallin proteins (α, β, and γ classes) account for more than 90% of total lens proteins.5 Lens transparency is suggested to rely on a short-range order of lens crystallin proteins.6 It is speculated that different crystallin isoforms are needed for the appropriate arrangement of the interfaces of membrane and cytoskeleton in lens fiber cells to ensure transparency and a high refractive index. α-Crystallins, consisting of αA and αB subunits, are members of the small heat shock protein family and have chaperone-like activity that prevents the aggregation of denatured proteins in vitro.7–9 Both β- and γ-crystallins are structural proteins that share a common Greek motif and that are extremely heat stable.10 β-Crystallins form dimers and higher oligomers, whereas γ-crystallins exist as monomers in the lens. Different γ-crystallin isoforms account for approximately one third of total lens proteins.11 Identification of γN-crystallin suggests an evolutionary link between β- and γ-crystallins.12 It is unknown whether β- and γ-crystallins play active roles in various cellular processes during differentiation and maturation of lens fiber cells. However, it is important to note that these crystallins are expressed in tissues other than the lens, suggesting that they may have other functions.12

γ-Crystallin isoforms A-F show approximately 77% to 97% identity at the protein level.11 Studies of recombinant, native, and mutant γ-crystallins have demonstrated significant variation in phase separation of different γ-crystallin isoforms and have provided some insights to explain how different γ-crystallin isoforms contribute to “cold cataracts” induced by lower temperature.13–16 Mutations that affect the stability and solubility of γ-crystallins can lead to cataracts in humans and mice,17–19 and some new studies suggest that γ-crystallins may have other important roles in the lens besides their role as passive structural components.12–15

Here we report a severe nuclear cataract caused by a new point mutation of γB-crystallin. We have found that this mutation specifically altered the subcellular distribution of γ-crystallin in mature fiber cells and led to unique cellular and biochemical changes that resulted in a dense nuclear cataract. This work provides some new information about how γB-crystallin is needed for the appropriate subcellular arrangement of proteins in lens mature fiber cells.

MATERIALS AND METHODS

Mouse Breeding and Causative Gene Identification

Mouse care and breeding were performed according to the Animal Care and Use Committee (ACUC)-approved animal protocol (UC Berkeley) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mouse pupils were dilated with 1% atropine and 1% phenylephrine before the eyes were examined for lens clarity by a slit lamp.
Histology, Immunohistochemistry, and Western Blotting

**Histology.** Enucleated eyeballs opened at the anterior chamber or posterior vitreous were immersed in a fixative solution containing 2% glutaraldehyde and 2.5% formaldehyde in 0.1 M cacodylate buffer (pH 7.2) at room temperature for 5 days. Samples were postfixed in 1% aqueous OsO₄ and then dehydrated through graded acetone. Samples were embedded in eponate 12-arylute 502 resin (Ted Pella, Redding, CA). Lens sections (1 μm thick) across the equatorial plane were collected on glass slides and stained with toluidine-blue. Bright-field images were acquired through a light microscope (Axiovert 200; Carl Zeiss, Oberkochen, Germany) with a digital camera.

**Immunohistochemistry.** A previously described procedure was used for preparing lens cryosections for immunohistochemical staining. A laser confocal microscope (Leica, Wetzlar, Germany) was used to collect fluorescent images.

**Western Blotting.** Lens total proteins were prepared by homogenizing enucleated fresh lenses that were weighed and homogenized directly in the sample buffer (60 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.001% bromophenol blue). Equal volumes (20 μL) of samples were loaded on a 12.5% SDS-PAGE gel for separation, and separated proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). Lens crystallin proteins were detected by Western blotting with rabbit polyclonal antibodies against α- and γ-crystallins (generously provided by Joseph Horwitz at University of California at Los Angeles), β-crystallin (generously provided by J. Samuel Zigler at the National Eye Institute), and a mouse monoclonal antibody against β-actin (Sigma, St. Louis, MO). More than three sets of lens protein samples of different mice were examined, and representative data were shown.

**Ion Concentration Measurement**

Lens ion concentration was measured by inductively coupled plasma-optical emission spectrometry (ICP-OES) from a core facility at the University of California, San Diego. The method was described previously. Lenses were dissected from postnatal day (P)10 wild-type, heterozygous and homozygous mutant mice and then immediately subjected to vacuum drying for 48 hours. Dry lenses were weighed and solubilized in 500 μL nitric acid (33.5—35%; Fisher Scientific, Pittsburgh, PA) for 12 hours at 37°C with shaking and then diluted with water into 3 mL. Samples were further diluted to reach ion concentrations of 20 ppb to 1 ppm for measurement. According to the estimated sample ion concentration, a series of standards was made. Ion concentrations were determined by their intensities acquired by the instrument. Measurement error was approximately 5%. Ion concentrations were normalized by lens dry weight.

**RESULTS**

A Dominant Nuclear Cataract Caused by the γB-S11R Mutation

We identified a dominant cataract from a spontaneous mouse mutant line (Nm3062) in the A/J strain background by slit lamp examination. Heterozygous mice displayed hazy nuclear cataracts, whereas homozygous mice developed dense nuclear cataracts at weaning age (Fig. 1A). Further examination of enucleated fresh lenses revealed that this nuclear cataract could be obviously visualized in mutant mice at P7, but not at P1. Mutant lenses also displayed a unique ringlike structure—located at approximately 220 to 350 μm away from the lens capsule—that caused abnormal light scattering. Homozygous mutant mice developed dense nuclear cataracts with full penetration in the A/J strain background or in mixed strain backgrounds, including C57BL/6J or 129 strains. Fifty-four backcross mice between Nm3062 mutant and wild-type CAST/Ei mice underwent phenotyping and genotyping for genomewide linkage analysis. This mutation was mapped to a region between the D1Mit19 and D1Mit21 markers on mouse chromosome 1. A cluster of γ-crystallin genes (CrygA-F) was located in this region. DNA sequence analysis of these γ-crystallin genes verified an A-to-C point mutation of the 11th codon of CrygB, which resulted in a substitution of serine with arginine at that codon in γB-crystallin (γB-S11R; Fig. 1B). No mutation was detected in other γ-crystallin genes.

**Normal Peripheral Fiber Cells and Disintegrated Inner Fiber Cells in the γB-S11R Mutant Lenses**

Toluidine blue-stained lens histology sections were prepared from neonatal mice (Fig. 2). These histologic sections showed that peripheral fibers developed normally at the bow region of P3 mutant lenses. However, inner fiber cells of heterozygous γB(S11R/+) and homozygous γB(S11R/S11R) lenses displayed uneven toluidine blue staining with abnormal darkly stained areas. Disintegrated fiber cells appeared only in the core of homozygous γB(S11R/S11R) lenses, whereas lens peripheral fiber cells displayed relatively normal morphology.

The periphery of γB(S11R/S11R) mutant lenses remained transparent even in older mice (Fig. 1A). Representative histologic data of lens peripheral fibers between wild-type and γB(S11R/S11R) mutant mice at P21 were shown (Fig. 2B). Lens cross-sections revealed that peripheral fiber cells of P21 γB(S11R/S11R) lenses remained relatively normal, similar to those of P21 wild-type lenses. Therefore, histologic data confirmed that altered inner fiber cells were correlated to the nuclear cataract phenotype caused by the γB-S11R mutation.
that, unlike P21 gated from actin filaments (Fig. 3A). Further studies of P7 and adjacent to the cell boundary of inner fibers and were segregated in lens fibers. Immunostaining of γB(S11R/S11R) lenses displayed uneven staining (arrowhead), and only γB(S11R/S11R) lenses revealed disintegrated fiber cells (arrow). (B) Lens cross-sections revealed that peripheral fiber cells of P21 γB(S11R/S11R) lenses remained relatively normal, similar to those of P21 wild-type lenses. Scale bars: (A) 10 μm; (B) 20 μm.

FIGURE 2. Histology of P3 and P21 wild-type and γB-S11R mutant lenses. (A, left) P3 whole lens sections. Scale bar, 100 μm. Right: high-magnification images of lens bow and inner regions. Bow regions of wild-type (WT) and mutant lenses displayed normal morphology. However, inner regions of γB(S11R+/+) and γB(S11R/S11R) lenses displayed uneven staining (arrowhead), and only γB(S11R/S11R) lenses revealed disintegrated fiber cells (arrow). (B) Lens cross-sections revealed that peripheral fiber cells of P21 γB(S11R/S11R) lenses remained relatively normal, similar to those of P21 wild-type lenses. Scale bars: (A) 10 μm; (B) 20 μm.

The Aggregation of γ-Crystallins Adjacent to the Cell Boundary and the Absence of F-Actin in Inner Fiber Cells

Based on the fact that the γB-S11R mutation leads to a dense nuclear cataract different from the lamellar cataract caused by the γB-I4F mutation, as reported previously,17 we hypothesize that, unlike γB-I4F, γB-S11R mutant proteins trigger a unique event to disrupt inner fiber cells to cause nuclear cataracts. To evaluate this hypothesis and to understand how mutant proteins lead to the disruption of inner fiber cells in γB(S11R/S11R) lenses, we examined the cellular distribution of γ-crystallins in lens fibers of wild-type and mutant lenses. γ-Crystallins account for approximately one third of total proteins in lens fibers. Immunostaining of γ-crystallin revealed uniformly distributed signal in peripheral and interior fiber cells of wild-type lenses (Fig. 3A). γ-Crystallins were also uniformly stained in peripheral fiber cells of γB(S11R/S11R) mutant lenses. However, γ-crystallin aggregates were detected adjacent to the cell boundary of inner fibers and were segregated from actin filaments (Fig. 3A). Further studies of P7 and P21 γB(S11R/S11R) lenses revealed that actin filaments completely disappeared in inner fiber cells, starting at approximately 220 to 350 μm away from the lens capsule (Fig. 3B). Thus, these results indicate that γB-S11R mutant proteins lead to the formation of unique γ-crystallin aggregates and the disruption of actin filaments in inner fiber cells. The loss of F-actin staining in the lens section (Fig. 3B) seems to be correlated with the appearance of the abnormal ring, peripheral to the dense nuclear cataract in γB(S11R/S11R) lenses (Fig. 1A).

FIGURE 3. Immunostaining of γ-crystallins and actin filaments in lens fiber cells. (A) P1 wild-type and homozygous γB(S11R/S11R) lens frozen sections were stained with anti-γ-crystallin antibody (green) and rhodamine-phalloidin (red). Boxes: selected areas that show separated and merged fluorescent images of γ-crystallin and F-actin in lens inner fiber cells. White arrows: γ-Crystallin aggregates adjacent to cell boundaries. White arrowbeads: F-actin. (B) Rhodamine-phalloidin-stained frozen sections of P7 and P21 wild-type and γB(S11R/S11R) lenses. Actin filaments disappeared in inner fiber cells of γB(S11R/S11R) mutant lenses, starting at approximately 350 μm from the lens capsule in the P7 sample and approximately 200 μm in the P21 sample (white arrowbeads). Scale bars, 50 μm.

Nuclear Cataracts and the Degradation of Crystallin Proteins

Morphologic data suggest that dense nuclear cataracts are associated with disintegrated inner fiber cells. To determine whether protein degradation occurred in these disintegrated lens fiber cells, we used Western blotting to analyze crystallins in wild-type and mutant lenses. Cleaved forms of different crystallins (αA, αB, β, γ) were detected in P21 mutant lenses but not in wild-type lenses. Moreover, cleaved αB- and γ-crystallins were detected in P7 mutant lenses (Fig. 4). Homozygous mutant lenses contained more cleaved crystallins than heterozygous mutant lenses. There was no detectable cleavage of αB-crystallin in P1 mutant lenses (data not shown). Thus, these Western blotting results indicated that the severity of the nuclear cataract was associated with the amount of cleaved crystallins (α and β/γ) in mutant lenses.
Elevated Calcium Concentration in Mutant Lenses

Activation of calcium-dependent proteases in mouse lenses often results in crystallin degradation. An influx of extracellular calcium into inner fiber cells could activate calcium-dependent proteases. We hypothesized that elevated calcium levels lead to increased crystallin degradation, resulting in severe nuclear cataracts in γB(S11R/S11R) mutant lenses. Therefore, we examined the total amount of ions, including calcium, magnesium, sodium, and potassium, in lenses by ICP-OES. The total calcium level was increased approximately fourfold in γB(S11R/S11R) lenses compared with wild-type lenses, whereas sodium, magnesium, and potassium levels were only slightly increased in γB(S11R/S11R) lenses. Calcium concentration was also increased in heterozygous γB(S11R/+ ) mutant lenses (Fig. 5). This result confirmed that crystallin degradation likely resulted from an activation of calcium-dependent proteases in γB-S11R mutant lenses. Furthermore, the degree of calcium elevation and the amount of cleaved crystallins were correlated with the severity of the nuclear cataract in γB-S11R mutant lenses.

**DISCUSSION**

This work provides at least part of the molecular mechanism for how the γBS11R mutation leads to a nuclear cataract. Histologic and biochemical data indicate that the dense nuclear cataract caused by this mutation is related to the degeneration of inner fiber cells. Fiber cell degeneration is correlated with an increase of cleaved crystallin proteins and a disruption of actin filaments. Specific aggregation of γ-crystallins in γB-S11R mutant lenses is likely to be one of the early events that trigger downstream changes, such as the loss of actin filaments and the elevation of calcium concentration. Activation of calcium-dependent proteases and disruption of cytoskeletal structures directly contribute to the degeneration of inner fiber cells, which lead to severe nuclear cataracts. However, some important questions remain to be answered. First, how do γB-S11R mutant proteins cause the aggregation of γ-crystallins adjacent to the cell boundary of inner mature fiber cells? Second, what leads to elevated calcium concentration? Third, how do actin filaments undergo disassembly? One possible explanation is that γ-crystallin aggregates disrupt or damage membrane-cytoskeletal structures to increase the influx of extracellular calcium, and elevated intracellular calcium subsequently activates calcium-dependent proteases, such as calpains, leading to crystallin degradation.

γ-Crystallin mutations are among the most common causes of hereditary cataracts in humans and mice. Studies of several mutant γ-crystallin proteins suggest that a decrease in the stability, solubility, or both of mutated proteins facilitates abnormal protein aggregates in the cytosol of fiber cells to cause distinct cataracts, such as “crystal-like” or lamellar cataracts. Others have also found that mutant γ-crystallin proteins can form nuclear aggregates in differentiating fiber cells to inhibit the denucleation process, leading to severe cataracts with ruptured lenses in mice. Therefore, nonspecific protein aggregation of mutated γ-crystallin proteins with reduced solubility or stability is not sufficient to explain how different types of cataracts can result from one particular mutation. Our experimental evidence suggests that mutated γ-crystallin proteins may perturb other specific protein–protein interactions or important cellular events during fiber cell maturation that lead to distinct types of cataracts. Different types of cataracts often result from a combination of a specific gene mutation and other genetic modifier(s).

The distribution of wild-type or mutant γB-crystallin proteins in the mouse lens has not been precisely determined. The γB-crystallin transcript is predominantly expressed in lens fibers, and the proportion of γB (and γC) proteins is reduced in the lens as mice age. The γB-I4F mutant protein is less heat stable in vitro, binds to α-crystallin in lens homogenates, and forms cytosolic aggregates in vivo. The γB-I4F mutation results in a lamellar cataract (from lens deep cortex to the nucleus), whereas the γB-S11R mutation causes an abnormal cortical ring and a dense nuclear cataract. Therefore, γB-cryst-

![Figure 4](Image)

**Figure 4.** Western blotting of lens crystallin proteins. Compared with wild-type (WT) lenses, cleaved αB- and γ-crystallins were detected in P7 mutant lenses (arrowheads). In P21 mutant lenses, cleaved forms of all crystallins (αA, αB, β, γ) were detected (arrows). Total amount of β-actin remained unchanged between P7 and P21 wild-type and mutant lenses.

![Figure 5](Image)

**Figure 5.** Total amounts of calcium, magnesium, sodium, and potassium in the lens measured by ICP-OES. Compared with WT lenses, calcium concentration was slightly increased in γB(S11R/+ ) lenses; total calcium level was increased approximately fourfold in γB(S11R/ S11R) lenses, whereas sodium, magnesium, and potassium levels were only slightly increased. These differences were statistically significant (n = 3; P < 0.05).
tallin is likely to be highly synthesized in differentiated fiber cells of the deep cortex. These two point mutations likely perturb the properties or functions of yβ-crystallin in different ways. We predict that additional factors, present only in mature fiber cells of lens nucleus, are required for yβ-S11R mutant proteins to trigger γ-crystallin aggregation adjacent to the cell boundary and to disrupt calcium homeostasis and the cytoskeleton in inner mature fiber cells.

Calcium homeostasis is crucial for lens transparency in humans and rodents.1,2,3 Mouse lenses are even more sensitive to calcium levels because of the presence of calcium-dependent proteases, such as calpains, especially the calpain-3 isoform (Lp82).34,35 Elevated intracellular calcium levels activate calpains that cleave α-, β-, and γ-crystallin proteins.54-56 Interestingly, the yβ-S11R mutation disrupts calcium homeostasis in the lens and causes activation of calcium-dependent degradation of crystallin proteins. Although β- and γ-crystallins lack a typical calcium binding site, recent studies suggest that the Greek key motif is directly involved in calcium binding in vitro.38-40 It is unclear whether γ-crystallin proteins regulate intracellular calcium level in lens fiber cells. However, it is important to further investigate how the yβ-S11R mutation perturbs calcium homeostasis in the lens.41,42

In summary, this work further supports the new hypothesis that mutant crystallins can cause cataracts by selectively perturbing protein–protein interactions, distinct cellular events during fiber cell maturation, or specific cellular structures of lens mature fibers. This differs from an old hypothesis that cataracts result from light scattering caused by high molecular weight aggregates formed by mutant proteins. However, it is unclear whether γ-crystallin affects lens transparency. It is unclear whether γ-crystallin affects self-protein–protein interactions or its interactions with other proteins in the lens. Further studies, such as characterisation of the solubility and stability of its recombinant proteins in vitro, determination of subcellular distribution of yβ-S11R protein in transfected cells, and identification of yβ-S11R interacting factor(s), may provide additional mechanistic information to explain the differences between yβ-S11R and yβ-F4 mutations and to elucidate the intermediate steps regarding how yβ-S11R mutant proteins lead to nuclear cataracts.

References


