Altered Chaperone-like Activity of α-Crystallins Promotes Cataractogenesis*

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Despite the enormous number of studies demonstrating changes in the chaperone-like activity of α-crystallins in vitro, little is known about how these changes influence life-long lens transparency in vivo. Using the γB-crystallin I4F mutant protein as a target for αA-crystallins, we examined how cataract phenotypes are modulated by interactions between α-crystallins with altered chaperone-like activities and γB-I4F proteins in vivo. Double heterozygous α-crystallin knock-out αA(+/−) αB(+/−) mice with a decreased amount of α-crystallins were used to simulate reduced total α-crystallin chaperone-like activity in vivo. We found that triple heterozygous αA(+/−) αB(+/−) γB(I4F/+ ) mice developed more severe whole cataracts than heterozygous γB(I4F/+ ) mice. Thus, total chaperone-like activity of α-crystallins is important for maintaining lens transparency. We further tested whether mutant αA-crystallin Y118D proteins with increased chaperone-like activity influenced the whole cataract caused by the γB-I4F mutation. Unexpectedly, compound αA(Y118D/+) γB(I4F/+) mutant lenses displayed severe nuclear cataracts, whereas the lens cortex remained unaffected. Thus, the synergistic effect of αA-Y118D and γB-I4F mutant proteins is detrimental to the transparency only in the lens core. α-Crystallins with different chaperone-like activities are likely required in the lens cortex and nucleus for maintaining transparency.

The eye lens is a transparent organ composed of bulk elongated fiber cells and a monolayer of epithelial cells covering the anterior surface (1–3). Ninety percent of all lens proteins are crystallins, which are subdivided into three classes, α, β, and γ. Lens transparency is believed to depend on the short-range order of crystallin proteins and the appropriate interactions among crystallin and non-crystallin proteins (4, 5). During aging and cataractogenesis, changes in lens protein interactions may result in high molecular weight aggregates that scatter light (6). Protein aggregation can also disrupt normal protein functions and/or create new pathological functions in the lens. Mechanisms for regulating the interactions among crystallin and non-crystallin proteins are not well understood.

α-Crystallins, consisting of αA- and αB-crystallin subunits, exist as polydispersed heteromeric aggregates in the lens. Long lens transparency is hypothesized to rely on the chaperone-like function of α-crystallins that acts as a “sink” for binding to denatured proteins to prevent abnormal protein aggregation induced by various risk factors (7). The chaperone-like activity of α-crystallins can be measured in vitro based on their ability to prevent heat or chemically induced, nonspecific protein aggregation of target proteins such as insulin and α-lactalbumin (8–13). Recent genetic, structural, and biochemical studies have revealed many new crystallin gene mutations, significantly improved the structural information of α- and γ-crystallin proteins, and elucidated various altered biochemical properties of α- and γ-crystallin mutant proteins that cause cataracts in humans and in mice (14–17).

Studies of α-crystallin mutations that cause cataracts have revealed altered chaperone-like activity of recombinant mutant α-crystallins in vitro. Because most α-crystallin mutant proteins display a decrease in chaperone-like activity in vitro, it is generally accepted that decreased chaperone-like activity of α-crystallin mutants is one of the mechanisms for cataract formation (11, 13, 18–21). However, several recent studies have indicated that mutant α-crystallins with increased chaperone-like activity also cause cataract formation (9, 22, 23). Thus, proper chaperone-like activity of α-crystallin proteins is probably required for maintaining lens transparency. The mechanistic difference between cataracts caused by reduced or increased chaperone-like activity of α-crystallin remains unknown. It is often difficult to interpret the results of the chaperone-like activity assays in vitro because these tests utilize non-lens proteins rather than native lens target proteins as binding substrates. The regulation of chaperone-like activity of α-crystallin in vivo is unclear due to the lack of a method that can be used to directly evaluate chaperone-like function during lens development or aging.

We reported previously that the mutant γB-I4F crystallin protein is a target protein for wild-type α-crystallins in vitro and in vivo (24). The γB-I4F mutation causes a dominant cataract, and homozygous lenses develop a much denser opacity than heterozygous lenses (24). Gel filtration analysis of lens homogenates shows that γB-I4F mutant proteins form high molecular weight aggregates with α-crystallins (24). Increased water-insoluble proteins are associated with cataract formation in γB-I4F lenses. These results indicate that endogenous α-crystallin proteins are unable to prevent the abnormal protein aggregation triggered by a large amount of γB-I4F mutant proteins in the lens. The more severe cataract of homozygous mutant mice is caused by the dosage effect of γB-I4F mutant proteins. Therefore, the γB-I4F mutant protein is...
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probably an appropriate target protein in vivo to test the effect of altered chaperone-like activity of α-crystallins in cataract formation. Decreased chaperone-like activity in the lens can presumably be recapitulated in vivo by using α-crystallin heterozygous knock-out mice because these mice develop clear lenses despite a decrease in the amount of α-crystallins (25, 26). Increased chaperone-like activity in the lens can be reproduced by the αA-Y118D mutant protein with increased chaperone-like activity in vitro, which we reported recently (9).

In this study, we used the γB-14F mutant protein as an in vivo target of α-crystallins to determine how altered α-crystallins affect lens transparency. Cataract severity has been previously (21, 27). Double heterozygous California, Berkeley). The generation and genotyping of A(Y118D/Y118D) and B(I4F/I4F) mice were described previously (25, 26). Increased chaperone-like activity in the lens can be recapitulated for qualitative (not quantitative) examination of the protein compositions in the peaks. Cloning and Expression Plasmids—Lens total RNA was isolated from wild-type and homozygous αA(Y118D/Y118D) or γB(I4F/I4F) mutant lenses with TRIzol reagent (Invitrogen). Total RNA was used to generate cDNA using the Superscript first-strand synthesis system for the RT-PCR kit (Invitrogen).

Coding region inserts were amplified using the Platinum® Pfx DNA polymerase kit (Invitrogen). Primers used to amplify the αA inserts were 5′-GGGAGCATGCTTCACCTCCACCCATGCCG, and primers used to amplify the γB inserts were 5′-GCGAATTCAGATGGAAGACGCCTCCACCCACCC-AGACGTGGGAGCAGGTCCACCTCCACCCATGCCG, and primers used to amplify the γB inserts were 5′-GCGAATTCAGATGGAAGACGCCTCCACCCACCCACCC-

Experimental Procedures

Mice—This study followed the Association for Research in Vision and Ophthalmic Statement for the Use of Animals in Ophthalmic and Vision Research and an Animal Care and Use Committee-approved animal protocol (University of California, Berkeley). The generation and genotyping of αA(Y118D/Y118D) and γB(I4F/I4F) mice were described previously (21, 27). Double heterozygous αA(Y118D/+γB(I4F/−) mice were generated by intercrossing αA(Y118D/Y118D) mice with γB(I4F/I4F) mice, and triple heterozygous αA(+/−) αB(+/−) γB(I4F/+) mice were generated by intercrossing αA(+/−) αB(+/−) γB(I4F/+) mice with γB(I4F/I4F) mice. The αA(+/−) αB(+/−) γB(I4F/+) mice were a generous gift from Dr. Eric Wawrusek at NEI (28). The γB(I4F/+) heterozygous mice were generated by crossing homozygous γB-14F mice with C57BL wild-type mice.

Expression, Purification, and in Vitro Binding of Recombinant Wild-type and Mutant αA and γB Proteins—Mouse wild-type αA, mutant αA-Y118D, wild-type γB, and mutant γB-I4F cDNAs were subcloned into a bacterial expression plasmid and prepared as described previously (8, 24, 29). Recombinant proteins that were exclusively in the inclusion bodies were solubilized in 8 M urea followed by stepwise buffer dialysis as described previously (30). For the in vitro binding analysis, 0.2 mg of each recombinant protein was mixed and heated to 45 °C for 30 min before being loaded into the AKTA FPLC system (GE Healthcare). These peaks were collected in different tubes with different elution volumes. Equal volumes of eluted solutions (20 μl) were mixed with loading buffer and loaded onto a 12.5% SDS-polyacrylamide gel for separation. Proteins were detected by Coomassie Blue staining for qualitative (not quantitative) examination of the protein compositions in the peaks.

Cloning and Expression Plasmids—Lens total RNA was isolated from wild-type and homozygous αA(Y118D/Y118D) or γB(I4F/I4F) mutant lenses with TRIzol reagent (Invitrogen). Total RNA was used to generate cDNA using the Superscript first-strand synthesis system for the RT-PCR kit (Invitrogen).
RESULTS

Recombinant α-A-Y118D Proteins Bind to Mutant γB-I4F but Not Wild-type γB-Crystallins in Vitro—To evaluate the interactions between wild-type or mutant α-A-crystallins and wild-type or mutant γB-crystallins, we performed in vitro binding assays using recombinant proteins produced from a bacterial expression system. Purified recombinant proteins were mixed and heated to induce protein aggregation before separation by gel filtration analysis. We tested three groups of mixed recombinant proteins: 1) mutant α-A-Y118D with mutant γB-I4F, 2) α-A-WT with mutant γB-I4F, and 3) mutant α-A-Y118D with γB-WT. Eluted proteins were separated on SDS-polyacrylamide gels. We found that mutant α-A-Y118D proteins bound to a large amount of mutant γB-I4F proteins (Fig. 1A, peak 1, and B, lane 1) and did not bind to wild-type γB-crystallins (Fig. 1A, peak 4, and B, lane 4). In comparison, wild-type α-A-crystallins bound to a small amount of γB-I4F (Fig. 1A, peak 3, and B, lane 3), and γB-I4F mutant proteins alone also formed a small amount of high molecular weight aggregates (Fig. 1A, peak 2, and B, lane 2). The small shoulder in peak 1 may be similar to that of peak 2. Compared with wild-type α-A-crystallin, mutant α-A-Y118D proteins probably have increased binding affinity for γB-I4F mutant proteins. The lag in the elution of peak 3 from the column, relative to peaks 1 and 4, was probably due to the fact that the average molecular weight of α-A-Y118D mutant protein aggregates was larger than that of the wild-type α-A-crystallin protein aggregates (9). We further examined the interaction between mutant α-A-Y118D and γB-I4F crystallins in transfected cells.

Mutant α-A-Y118D and γB-I4F Proteins Form Aggregates in Co-transfected Cells—Expression plasmids for wild-type α-A-crystallin, mutant α-A-Y118D crystallin tagged with GFP, wild-type γB-crystallin, or mutant γB-I4F crystallin tagged with RFP were transfected individually into a mouse immortalized lens epithelial cell line (Fig. 2A). GFP-tagged wild-type α-A-crystallins alone were uniformly distributed in the cytoplasm, whereas mutant α-A-Y118D proteins alone formed cytoplas-
mic protein aggregates (Fig. 2B). Similarly, RFP-tagged wild-type γB-crystallins alone were uniformly distributed in cells without aggregates, whereas mutant γB-I4F proteins alone formed cytoplasmic aggregates (Fig. 2B). Co-transfection of wild-type and mutant crystallins revealed that αA-WT and γB-WT proteins maintained uniform protein distribution in the cytosol, whereas co-expression of αA-WT and γB-I4F proteins caused large aggregates (Fig. 3, A and B). In addition, co-expression of αA-Y118D and γB-WT showed that only a very small amount of γB-WT proteins was co-localized with αA-Y118D aggregates (Fig. 3C). In contrast, a large amount of γB-I4F mutant proteins was present in the αA-Y118D aggregates (Fig. 3D). These results indicate that mutant γB-I4F is truly a target protein for both αA-WT and mutant αA-Y118D in cells, as suggested by the binding data in vitro. Thus, the γB-I4F mutant protein might serve as a target substrate in vivo to determine how the interactions between α-crystallins with altered chaperone-like activity and γB-I4F mutant proteins influence lens transparency.

Altered Chaperone-like Activity of α-crystallins Increases the Severity of Cataracts Caused by the γB-I4F Mutation—The αA(+/−) αB(+/−) double heterozygous knock-out mice, which display clear lenses like wild-type mice (data not shown), were used to reduce lens total chaperone-like activity of α-crystallins in vivo. The αA-Y118D heterozygous mutant mice, which develop mild nuclear cataracts (27), were used to evaluate the affect of αA-crystallin with increased chaperone-like activity. Thus, we generated αA(+/−) αB(+/−) γB(I4F/+ ) triple heterozygous mutant mice and αA(Y118D+)/+ γB(I4F/+ ) double heterozygous mutant mice and compared their lens phenotypes.

In contrast to the mild cataracts seen in γB(I4F/+ ) lenses, the αA(+/−) αB(+/−) γB(I4F/+ ) lenses had more severe cataracts, but increased opacity was less obvious in the lens core, as shown by lens pictures, densitometric surface plots, and linear plot profiles of these cataracts (Fig. 4). Quantitative measurements of light scattered from these cataracts further showed that the αA(+/−) αB(+/−) γB(I4F/+ ) lenses had a statistically significant increase in light scattering compared with γB(I4F/+ ) lenses (Fig. 5). We tried to evaluate cataract formation in younger mice. However, due to cold cataract formation in neonatal lenses (31) and the very mild cataract phenotype in triple heterozygous lenses, we were unable to obtain reliable evidence to determine whether the cataracts in triple heterozygous mice developed earlier or were more severe at neonatal stages.

Unexpectedly, the αA(Y118D+)/+ γB(I4F/+ ) double mutant lens had a dense cataract that was predominantly in the lens nucleus (Fig. 4). Light scattering measurements confirmed that compound mutant lenses scattered more light than γB(I4F/+ ) lenses (Fig. 5). The combination of the amount of light scattered by αA(Y118D+)/+ lenses and γB(I4F/+ ) lenses was about 50% of the amount of light scattered by αA(Y118D+)/+ γB(I4F/+ ) double heterozygous lenses. Therefore, the dense nuclear cataracts in double heterozygous mutant lenses likely resulted from enhanced interaction between αA-Y118D and γB-I4F mutant proteins.

**DISCUSSION**

This study provides direct in vivo evidence demonstrating that lens transparency is influenced by the interactions between α-crystallins and a native target substrate, γB-I4F crystallin. Although αA(+/−) αB(+/−) mice develop normal lenses, increased light scattering from cataractous αA(+/−) αB(+/−) γB(I4F/+ ) lenses indicates that the function of α-crystallin is impaired in αA(+/−) αB(+/−) mice. A normal amount of α-crystallins is important for lens transparency. It is well known that αA-crystallins undergo posttranslational modifications to alter the chaperone-like activity during lens
development or during aging of a normal lens (32). The precise regulation of the chaperone-like activity of H9251-crystallin and the proper binding affinity between H9251-crystallin and other lens proteins are likely required for establishing and maintaining lens transparency.

Increased binding affinity of H9251-A-Y118D mutant proteins to H9253-B-I4F mutant proteins probably generates more protein aggregates, leading to light scattering in the lens. These results reveal that the synergistic effect of H9251-A-Y118D and H9253-B-I4F mutant protein interactions is detrimental to the transparency only in the lens core. Thus, H9251-crystallins with different chaperone-like activity are likely needed in the lens cortex and nucleus for maintaining transparency. This work supports the notion that lens transparency may require high chaperone-like activity of H9251-crystallins in the lens cortex, but low chaperone-like activity of H9251-crystallins in the lens nucleus.

Aberrant Aggregates of α-A-Y118D Mutant Proteins in Cultured Cells—We have confirmed that the α-A-Y118D crystallin is a gain-of-function mutant that binds strongly to γB-I4F mutant proteins in transfected cells and in recombinant protein binding assays. Endogenous αA-crystallin proteins were not detectable in the immortalized mouse lens epithelial cells, whereas αB-crystallins could be detected by Western blotting (data not shown). Increased chaperone-like activity (or increased binding affinity) of αA-Y118D mutant proteins may cause abnormal αA-Y118D protein aggregation or binding to other endogenous proteins. It is possible that the αA-Y118D mutant protein by itself is unstable or causes precipitation of normal proteins within lens cells. Currently, it is unclear why αA-Y118D mutant proteins form aggregates in transfected cells and what the other proteins are in these aggregates. We confirmed that these αA-Y118D aggregates are not co-localized with the lysosome marker Lamp-1, the Golgi marker giantin, or the endoplasmic reticulum marker calreticulin (data not shown). For future studies, it will be very interesting to investigate the molecular basis for these aggregates and to determine whether ubiquitination is associated with the mutant protein aggregates.

**In Vivo Chaperone-like Activity of α-Crystallins Is Essential for Lens Transparency**—Previous studies were unable to prove the necessity for utilizing both alleles of αA and
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αB genes because αA(+/−) and αB(+/−) mice develop clear lenses despite a decrease in the amount of αA and αB proteins, respectively (25, 26). More severe cataracts in αA(+/−) αB(+/−) γB(14F/+ ) lenses indicate that α-crystallins expressed from both gene alleles are essential for maintaining lens transparency. Decades of in vitro studies have suggested that α-crystallins with chaperone-like activity may serve a protective function in the lens (7, 8). Recessive cataracts caused by αA-crystallin null mutations in humans and in mice indicate that αA-crystallin is essential for establishing and/or maintaining transparency in wild-type lenses (25, 33). However, α-crystallins have not been directly shown to prevent cataract formation caused by other mutations in vivo. Opacity differences between γB(14F/+ ) and αA(+/−) αB(+/−) γB(14F/+ ) lenses reveal the first in vivo evidence that α-crystallins suppress cataract formation induced by the unstable γB-14F mutant protein. Thus, γB-14F mutant proteins are a useful native “denatured” target protein for wild-type and mutant α-crystallins for in vitro and in vivo chaperone-like activity assays.

Previous studies of the γB-14F mutation suggested that mutant γB-14F proteins were less heat-stable (24). It is likely that an insufficient amount of α-crystallin proteins results in the aggregation of unstable γB-14F mutant proteins shown in Fig. 1A (H.W. peak 2). The molar ratio of α-crystallin multimers was much lower than that of γB-14F protein monomers in the in vitro binding assay. Presumably, an insufficient amount of α-crystallins also cause increased aggregation and insolubility of γB-14F mutant proteins in αA(+/−) αB(+/−) γB(14F/+ ) mutant lenses in vivo. Further experiments will be needed to elucidate the mechanisms for cataractogenesis in αA(+/−) αB(+/−) γB(14F/+ ) and αA(Y118D/+ ) γB(14F/+ ) mutant lenses. However, it is difficult to separate the effects of mutant proteins versus increased chaperone-like activity in vivo. It may be interesting to determine whether an extra amount of wild-type αA-crystallin, expressed from a transgenic mouse line, can inhibit cataracts caused by the γB-14F mutation.

Quantitative Light Scattering Measurements Accurately Determine Cataract Severity in Mouse Lenses—We have implemented and tested a quantitative method to determine light scattering in cataractous lenses among different mouse strains using a fiber-optic cable and spectrometer. Previous studies have used qualitative scoring methods that rely on the judgment of researchers or imaging the lens on a grid background (34). These methods cannot be used to quantify cataract severity. Without the light scattering intensity quantification, it would be difficult to demonstrate a significant difference between γB(14F/+ ) and αA(+/−) αB(+/−) γB(14F/+ ) lenses. Moreover, this new quantification method can be easily established in any laboratory and is an excellent tool for vision researchers to use to quantitatively study cataractogenesis in mice.

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FIGURE 5. Quantitative measurements of light scattering from compound mutant lenses reveals a statistically significant increase in light scattering caused by altered α-crystallin chaperone-like activity. A, the αA (+/−) αB (+/−) γB(14F/+ ) and αA(Y118D/+ ) γB(14F/+ ) lenses scattered more light, as measured by intensity over the visible wavelength, than γB(14F/+ ) and αA(Y118D/+ ) lenses. The wild-type lens represents the base line of the light scattering intensity. B, bar graph of the mean ± S.D. of normalized intensity for each genotype (at least six lenses). The intensity of scattered light was arbitrarily normalized based on 0% for wild-type lenses and 100% for αA(Y118D/+ ) γB(14F/+ ) lenses. The difference between γB(14F/+ ) and αA(Y118D/+ ) γB(14F/+ ) lenses and the difference between γB(14F/+ ) and αA(+/−) αB(+/−) γB(14F/+ ) lenses were statistically significant (p < 0.001).
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