Published in final edited form as:

Exp Eye Res. 2017 February ; 155: 85–90. doi:10.1016/j.exer.2017.01.005.

BetaB2-crystallin mutations associated with cataract and glaucoma leads to mitochondrial alterations in lens epithelial cells and retinal neurons

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Abstract

Crystallin proteins are the most prominent protein of the lens and have been increasingly shown to play critical roles in other tissues, especially the retina. Members of all 3 sub-families of crystallins, alpha-, beta- and gamma-crystallins have been reported in the retina during diabetes, traumatic injury and other retinal diseases. While their specific role in the retina is still unclear and may vary, beta-crystallin proteins have been shown to play a critical role in ganglion cell survival following trauma. We recently reported the correlation between a gene conversion in the betaB2-crystallin gene and a phenotype of familial congenital cataract. Interestingly, in half of the patients, this phenotype was associated with glaucoma. Taken together, these data suggested that the mutations we recently reported could have an impact on the role of betaB2-crystallin in both lens epithelial cells and retinal neurons. Consistent with this hypothesis, we show in the current study that the gene conversion leading to an amino acid conversion lead to a loss of solubility and a change of subcellular localization of betaB2-crystallin in both cell types. While the overall observations were similar in both cell types, there were some important nuances between them, suggesting different roles and regulation of betaB2-crystallin in lens cells versus retinal neurons. The data reported in this study strongly support a significant role of betaB2-crystallin in both lenticular and retinal ocular tissues and warrant further analysis of its regulation and its impact not only in cataract formation but also in retinal neurodegenerative diseases.

Mutations in crystallins, the most prominent protein in the lens, have long been known to lead to various forms of cataract. Mutations in β B2-crystallin (*CRYBB2*) have been

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Author Contributions. J.E.D. collected and analyzed data and wrote the manuscript. A.R. collected and analyzed data and edited the manuscript. S.J.G. collected and analyzed data and edited the manuscript. H.S.P. obtained funding, aided with data analysis, and edited the manuscript. P.E.F. obtained funding, designed the experiments, analyzed data and edited the manuscript.

Disclosures. No potential conflicts of interest relevant to this article were reported.

exclusively associated with autosomal dominant congenital cataracts, though the underlying mechanism remains unclear (Faletra et al., 2013; Hansen et al., 2009; Lou et al., 2009; Wang et al., 2009a; Weisschuh et al., 2012). In more recent years, both α - and β -crystallins have been shown to play critical roles in protection of retinal neurons in various retinal diseases. While α -crystallins play a key role in protecting retinal neurons from retinal diseases such as uveitis and diabetic retinopathy (Losiewicz and Fort, 2011; Rao et al., 2008), β -crystallins have been shown to play a critical role in regeneration of axonal retinal ganglion cells (Bohm et al., 2015; Fischer et al., 2008; Liedtke et al., 2007). Damage to retinal ganglion cells is a critical aspect of the pathophysiology of glaucoma (Kuehn et al., 2005), one of the most serious complications associated with congenital cataract surgery, with an incidence rate as high as 32% (Asrani and Wilensky, 1995; Bradford et al., 1994; Francois, 1979; Magnusson et al., 2000; Mills and Robb, 1994). A recent report demonstrated that mutations in the RNA granule component TDRD7, which causes autosomal recessive cataract, also causes glaucoma (Lachke et al., 2011). This study also showed that crystallins, including β B2-crystallin, are down-regulated in the lens of Tdrd7-null mutant mouse. We recently identified and reported a familial congenital cataract phenotype due to a gene conversion of β B2-crystallin that carries three variants, R145W, Q147R, and T150M (Garnai et al., 2014). Five of the ten family members presented with glaucoma concomitantly with congenital cataract. While this could reflect a putative role for betaB2-crystallin in glaucoma pathophysiology, the role of another gene co-segregating cannot be completely excluded at this time.

The β B2-crystallin polypeptide is a multi-domain protein containing four Greek key motifs, which oligomerizes into larger, functional complexes. Mutations associated with congenital cataract have been found in most regions of the protein, though ten of the fourteen reported mutations are located in Greek key motifs 3 and 4. Of the three amino acid conversions we recently identified, the first two are located in Greek key motif 3 while the third is in Greek key motif 4. Two cataract-associated mutations of β B2-crystallin found in Greek key motif 4, Q155X and W151C, have been shown to increase aggregation of β B2-crystallin protein (Chen et al., 2013; Liu and Liang, 2005). Indeed, crystallin mutations associated with cataract often lead to protein aggregation; however, little data has been reported for β B2-crystallin aggregation (Chen et al., 2013; Liu and Liang, 2005; Moreau and King, 2012a, b; Raju and Abraham, 2011). In addition, β B2-crystallin is also expressed in the retina and plays a key role in the protection of retinal neurons and the regeneration of their processes, critical aspects of the pathophysiology of glaucoma (Bohm et al., 2015; Prokosch et al., 2013; Thanos et al., 2012).

The purpose of this study was to analyze the effect of the gene conversion (hereafter referred to as “triple mutant”) we recently reported on the structure and function of β B2-crystallin protein. While independent studies have been performed, to date, very few attempts have been made to study the effect of the same crystallin mutations in both lens and retina (Graw et al., 2006). We investigated the solubility and subcellular localization of the triple mutant of β B2-crystallin corresponding to the gene conversion we recently reported (Garnai et al., 2014) in both lens cells and retinal neurons to evaluate the potential parallel regulation of crystallin protein functions in both ocular tissues.

The β B2-crystallin cDNA was generated by RT-PCR using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) using RNA from human retina and amplified using *CRYBB2*-specific primers. The PCR product was cloned into pGEM-T Easy, and then subcloned into pcDNA3.1⁺. Three mutations, c.433 C>T (p.R145W), c.440A>G (p.Q147R), and c.449C>T (p.T150M), were introduced into the wild type *CRYBB2*-carrying clone using the QuikChange Lightning kit (Agilent). Constructs were confirmed by sequencing.

Human lens epithelial (HLE) B-3 cells (ATCC) were maintained in Dulbecco's modification of Eagle's minimum essential medium (DMEM) with 20% fetal bovine serum (FBS) and 1% penicillin/streptomycin, while the retinal neuron (R28) cell line was maintained in DMEM with 10% FBS. HLE-B3 cells were transfected with the β B2-crystallin constructs using the X-tremeGENE 9 kit (Roche), while R28 cells were transfected using Lipofectamine 3000 (Thermo Fisher). Cells were harvested 48 hours after transfection for analysis in solubility assays. Additionally, prior to transfection, cells were plated on laminin-coated coverslips for analysis by immunofluorescence (IF). After plating, cells were transfected with β B2-crystallin constructs, and 24 hours after transfection, fixed using methanol/acetone (1:1) at -20°C . Transfection efficiency was optimized in order to obtain similar levels of expression for both WT and mutant plasmids.

To analyze β B2-crystallin solubility, cells were lysed in RIPA buffer (100mM Tris, pH 7.5, 3mM EGTA, 5mM MgCl_2 , 0.5% TritonX-100, 1mM PMSF, and 1X complete EDTA-free protease inhibitor cocktail (Roche)). Cell lysates were sonicated, and then spun at 13,000rpm for 10 minutes in a microcentrifuge. The supernatant was removed, and the pellet was resuspended in Urea buffer (10mM Tris, pH 7.5, 150mM NaCl, 5mM EGTA, 5mM MgCl_2 , 1mM DTT, 0.1% TritonX-100, 0.2mM PMSF, and 9M urea). Samples were separated by SDS-PAGE on a 4–12% gradient gel and analyzed by western blotting using primary antibody against β B2-crystallin (Santa Cruz Biotechnology).

To analyze the structure and localization of β B2-crystallin by IF, cells on coverslips were first incubated in blocking buffer containing 10% normal donkey serum, then stained with primary antibody against β B2-crystallin and either CoxIV (Cell Signaling), Lamp1 (Santa Cruz), EEA1 (Abcam) or LC3 (Novus), overnight at 4°C . Cells were then counterstained with secondary antibodies conjugated to the fluorophores AlexaFluor488 and AlexaFluor647 (Jackson laboratories). Finally, cells were stained with Hoechst nuclear dye (Life Technologies), and the coverslips were mounted on glass slides. Cells were imaged on a Leica SP5 confocal microscope and the images were analyzed using IMARIS software and ImageJ.

Since mutations in crystallins, which result in cataracts, often result in altered structure or solubility of the crystallin proteins, we began by analyzing the solubility of the β B2-crystallin triple mutant in lens cells (HLE-B3). We started by testing the specificity and sensitivity of the antibody against β B2-crystallin by transfecting those cells with the constructs made and comparing it to an ocular lens sample (See Figure in Dulle et al., submitted). We found that the β B2-crystallin triple mutant was largely insoluble in lens cells compared to the WT β B2-crystallin (Fig. 1A). This shift of mutant β B2-crystallin to the

insoluble fraction is characteristic of several disease-associated mutations of β B2-crystallin (Chambers and Russell, 1991; Sun et al., 2013). When we expressed the mutant β B2-crystallin in retinal neurons (R28 cells), we observed an even stronger shift in solubility. While in the lens cells 35% of the mutant protein remained soluble, the β B2-crystallin triple mutant was almost completely insoluble in retinal neurons (Fig. 1A).

To determine whether mutant β B2-crystallin was forming aggregates in the lens cells and retinal neurons, we expressed WT and mutant β B2-crystallin in both cell lines and performed IF on cells using an antibody against β B2-crystallin. While the WT β B2-crystallin displayed a diffuse pattern of fluorescence in both cell types, the β B2-crystallin triple mutant exhibited a punctate pattern of fluorescence, often characteristic of decreased solubility (Fig. 1B). Overall the primary results of IF were very consistent with the decreased solubility of the mutant β B2-crystallin we observed by western blot.

Upon further analysis of the images generated by IF, we found that the fluorescent pattern of the mutant β B2-crystallin was more congruent with that of proteins associated with subcellular compartments or intracellular vesicles than purely protein aggregates. Previous reports have shown that β B2-crystallin protein is oxidized and localized to the mitochondria in photoreceptors isolated from experimental autoimmune uveitis (EAU)-induced mice (Saraswathy and Rao, 2009, 2011). Of note, this finding could not be confirmed in the vitreous or aqueous humor of a rat model of uveitis (Pepple et al., 2015) in which β B2-crystallin levels were reduced. This difference is consistent with the fact that the proteome of the vitreous or aqueous humor do not always reflect that of the retina but could also be due to a difference in the timing of the analysis. To the best of our knowledge, these are the only reports demonstrating mitochondrial localization of β B2-crystallin, indicating how little is known about non-structural mechanisms of β B2-crystallin. To determine if the triple mutant β B2-crystallin localized to any subcellular compartments, we performed IF analysis, comparing the pattern of expression of the mutant β B2-crystallin with components of several subcellular compartments including mitochondria (Cox IV, Figure 2A–D), ER and Golgi (EEA1, Figure 2E–H), lysosomes and autophagosomes (Lamp 1 and LC3, data not shown). Only CoxIV, a subcellular marker for the mitochondria, showed significant co-localization with the β B2-crystallin mutant (Fig. 2C–D). While a punctate staining was also observed, no colocalization was observed between β B2-crystallin and either EEA1 (Fig. 2G–H), Lamp1 or LC3, suggesting that the insoluble proteins are not accumulating in these subcellular organelles. In the lens cells, the fluorescent pattern of β B2-crystallin overlaps with that of the mitochondrial marker, suggesting that the congenital cataract pathology associated with this triple mutant may correlate with aberrant localization to the mitochondria. Such a subcellular localization would support both a loss of function with this mislocalization impairing its normal structural function in the lens, but also potentially a toxic gain of function with potential alteration of mitochondrial function. This second hypothesis is supported by the difference of the pattern of CoxIV signal observed in the adjacent cells not overexpressing mutant β B2-crystallin (Fig. 2D, arrows). While in cells overexpressing WT β B2-crystallin the mitochondria form a clear network around the nuclei (Fig. 2B), in the cells overexpressing the mutant β B2-crystallin the mitochondria appear much smaller and disconnected (Fig. 2D). The triple mutant β B2-crystallin only partially co-localizes with the mitochondria in the retinal neurons (Fig. 2D), with the majority of the

triple mutant β B2-crystallin localized at or adjacent to mitochondria, while some remains insoluble in the cytoplasm. While there are some striking differences between the localization of the mutant β B2-crystallin in lens and retinal neuron cells, the mitochondrial pattern also appears to be different from the retinal neurons not overexpressing the mutant β B2-crystallin protein (Fig. 2D, arrows). We further performed cellular compartments fractionation experiments, which showed that while the WT protein was only cytosolic, a portion of the β B2-crystallin mutant protein was found in the mitochondrial fraction (Fig. 2I).

β B2-crystallin has been shown to have chaperone functions distinct from its structural roles (Zhang et al., 2001). To date, the most significant non-structural role for β B2-crystallin elucidated has been in promoting axonal regeneration in the retina. It has been long established that lens injury mitigates retinal ganglion cell loss and promotes retinal ganglion cell axon regeneration (Fischer et al., 2001; Fischer et al., 2000; Stupp et al., 2005; Wang et al., 2009b; Wong et al., 2006). As a substantial component of lens cells, crystallins were attractive candidates as the driving force underlying the protective effects of lens injury. Indeed, β B2-crystallin is secreted by cultured retinas during axonal regeneration and aids elongation in vitro when added to culture medium (Liedtke et al., 2007). Furthermore, intravitreal injections of purified β -crystallins induced a regenerative state in retinal ganglion cells after axotomy or optic nerve crush in rats, demonstrating strong evidence for a therapeutic use for β -crystallins (Fischer et al., 2008; Thanos et al., 2014). However, before β -crystallins can be used as therapy for human eye diseases or injuries, the underlying mechanisms need to be more properly investigated.

We demonstrate here that the cataract-associated gene conversion of β B2-crystallin has a multi-fold effect on its structure and function including (1) loss of solubility and (2) aberrant localization to the mitochondria in two different cell types. Since in the R28 cells we observe only partial localization to the mitochondria, we suggest that the exchange of 3 amino acids promotes aggregation of β B2-crystallin, which then leads to translocation to the mitochondria either directly or indirectly through increased interaction with mitochondrial proteins. Since β B2-crystallin has been shown to bind other proteins through interactions with the hydrophobic core (Fu and Liang, 2002; Takata et al., 2009), we don't yet know if the localization to the mitochondria is a result of an interaction with another protein, for example an apoptotic protein or another crystallin chaperone. Formation of protein aggregates often induces increased interactions with non-canonical partners. Mutations that increase the ability of β B2-crystallin to bind these proteins could, as a consequence, enhance aberrant localization to mitochondria. Whatever the case maybe, we tested the impact of this translocation on mitochondrial function by seeding cells in XF24 cell culture plates (Seahorse Bioscience, Billerica, MA, USA). Cells were transfected as described above and 24h later analyzed in a XF24 analyzer for oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). The translocation to the mitochondria clearly leads to a loss of mitochondrial function as demonstrated by reduced basal oxygen consumption rate in the mutant (290 ± 17) compared to empty vector (320 ± 17) or WT (337 ± 11) transfected HLE-B3 cells ($n=6$; $p<0.05$ for mutant compared to EV and WT, Fig. 2J).

Our data demonstrating mitochondrial localization of β B2-crystallin in both lens and retinal cells parallels the previous report showing increased expression of β B2-crystallin in the mitochondria of photoreceptors during the non-apoptotic phase of EAU (Saraswathy and Rao, 2009). In this report, the authors suggested that β B2-crystallin expression in the mitochondria may be beneficial as a way to prevent cell death during the early stages of EAU. Despite evidence that β B2-crystallin can promote axon regeneration, there is no real evidence that localization of β B2-crystallin to the mitochondria is due to a beneficial chaperone function. Indeed, our data suggests that translocation of β B2-crystallin, at least this specific variant, to the mitochondria may contribute to the pathological phenotype of cataracts. Aberrant localization of β B2-crystallin to the mitochondria may cause mitochondrial damage, loss of function, and subsequent induction of apoptosis. Indeed, this hypothesis would also be consistent with the lower transfection efficiency observed for the mutant when compared to WT, in both lens and retinal cells, suggesting a correlation between expression of the variant and cell toxicity. Our novel findings, while obtained in cell lines with their inherent limitations including variation of expression and morphology compared to the original cell type studied (Wang-Su et al., 2003), and thus requiring further validation in primary cells, strongly suggest that mutations of β B2-crystallin associated with cataract and glaucoma alter protein solubility and could cause detrimental mitochondrial localization resulting in both lens and retinal cell death. Given our findings, changes to the structural or chaperone functions of β B2-crystallin should be explored in more depth with an array of known disease-associated β B2-crystallin mutations.

Acknowledgments

This project and its authors were supported by an NEI T32-EY013934 training grant and an international retina research foundation (IRRF) postdoctoral fellowship (J.E.D.), a fellowship from the Dr. Werner Jackstaedt Foundation (A.R.) and NIH EY020895 (P.E.F.). This work utilized the Core Center for Vision Research funded by P30 EY007003 from the National Eye Institute.

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Figure 1. Mutations associated with early-onset cataracts alter the solubility of β B2-crystallin in lens cells and retinal neurons

(A) Solubility of wild-type (WT) and the triple mutant (R145W, Q147R, T150M) β B2-crystallin was measured in lens (HLE) and retinal (R28) cells. Lysates from cells transfected with either WT, mutant β B2-crystallin or the corresponding empty vector (EV) were separated into soluble (S) and insoluble (I) fractions by centrifugation and analyzed by western blot. A representative image of the signal obtained for β B2-crystallin in each cell-type as well as the quantification of 3 independent replicates are shown (** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ statistically significantly different from WT using ttest). (B)

Cellular expression and aggregation patterns of WT and the triple mutant (R145W, Q147R, T150M) β B2-crystallin were analyzed by immunofluorescence (IF). Representative images of the IF signal obtained for β B2-crystallin (green) and Hoescht (nucleus, blue) are shown for each cell-type. Scale bar = 10 μ m.

Figure 2. Mutations associated with early-onset cataracts cause mislocalization of β B2-crystallin to the mitochondria and mitochondrial dysfunction

Subcellular localization of the β B2-WT-crystallin (A, B, E, F) and the triple mutant (R145W, Q147R, T150M) β B2-crystallin (C, D, G, H) were assessed by co-IF with markers for various specific cellular organelles in lens (HLE) and retinal (R28) cells. Cells were stained for β B2-crystallin (green), and CoxIV (A–D) or EEA1 (E–H, red) and counterstained with Hoechst (nuclei, blue). Higher magnification images of relevant regions of the cells (marked by a dashed square) are shown for each co-staining. Images of the mitochondrial staining of cells overexpressing (D, insert 1) or not overexpressing (D, insert

2) the triple mutant (R145W, Q147R, T150M) β B2-crystallin are shown (arrows points to the difference of the mitochondrial pattern). The higher magnification images clearly show the co-localization between β B2-crystallin and CoxIV indicating localization of triple mutant β B2-crystallin to the mitochondria. Little to no colocalization was detected for any of the markers of the other organelles tested. Immunoblotting of subcellular fractions confirms an enrichment of the triple mutant β B2-crystallin, but not of the WT, in the mitochondrial fraction in HLE cells (I), associated with mitochondrial respiratory dysfunction demonstrated by lower oxygen consumption rate in β B2-crystallin mutant compared to WT β B2-crystallin or EV transfected HLE cells (J). Scale bar = 10 μ m (for A, C, E and G) and 2 μ m (for B, D, F and H).