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A comprehensive analysis of the expression of crystallins in mouse retina

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Purpose: Crystallins are expressed at high levels in lens fiber cells. Recent studies have revealed that several members of the α, β, and γ-crystallin family are also distributed in many non-lens tissues, though at lower levels. We observed that the use of retinal RNA as target for both custom I-Gene microarrays and Affymetrix GeneChips revealed significant expression of many crystallin genes. This prompted us to undertake a comprehensive investigation to delineate the baseline expression of crystallin genes in the adult mouse retina.
Methods: Quantitative RT-PCR was carried out using gene specific primers (derived from the mouse genomic sequence) for each crystallin gene. Immunofluorescence studies using frozen sections of the mouse retinas were performed with crystallin-specific antibodies. Retinal lysates were analyzed by immunoblotting using antibodies specific to αA and αB crystallins and those produced against total β-crystallin and γ-crystallin fractions of bovine lenses.
Results: Microarray analysis followed by quantitative RT-PCR revealed that mouse retinal cells express transcripts for 20 different members of the crystallin gene family; these are αA, αA-INS, αA-nov1, αB, βA1, βA2, βA3, βA4, βB1, βB2, βB3, γA, γC, γD, γE, γF, γS, μ, ζ, and λ-crystallin. The gene products of αA, αB, β-, and γ-crystallins are detected in the outer and inner nuclear layers of the retina by immunofluorescence analysis. In addition, αB and β-crystallins are detected in the photoreceptor inner segments. Retinal expression of these proteins was further confirmed by immunoblot analysis. Interestingly, our studies also showed a significant animal-to-animal variation in the expression level of some of the crystallins.
Conclusions: Our results establish the expression of many crystallins in the adult mouse retina. Detection of crystallins in the retinal nuclear layers, though surprising, is consistent with their proposed role in cell survival and genomic stability. We suggest that crystallins play vital functions in protecting retinal neurons from damage by environmental and/or metabolic stress.

Crystallins constitute a diverse group of proteins that are expressed at high concentrations in the differentiated lens fiber cells and augment the refractive power of the transparent lens tissue [1,2]. In vertebrates, three major classes of crystallins, α, β, and γ, accumulate in the lens in a spatially and temporally regulated manner [3-5]. Their expression increases dramatically during differentiation of lens epithelial cells into fibers [1]. The two α-crystallins (αA and αB) belong to the small heat shock protein family of molecular chaperones and appear very early during mouse embryonic development [6,7]. Members of the β/γ-superfamily, which include β-crystallins (βA1/A3, βA2, βA4, βB1, βB2 and βB3) and γ-crystallins (γA-F, and γS, formerly βS), are related to microbial proteins induced by physiological stress [8,9]. In addition, a growing number of crystallins (known as taxon-specific enzyme-crystallins) are expressed at relatively high levels in the lens but only in selected species; these proteins include μ, ζand λ-crystallins that are closely related to metabolic enzymes ornithine cyclodeaminase, NADPH:quinone oxidoreductase and hydroxyl CoA dehydrogenase, respectively [3,10].

Originally considered to be static, abundant proteins providing transparency to the lens, it is now generally accepted that crystallins were selected from proteins with entirely different non-lens roles and are retained in multiple tissues of the same organism [6]. Bhat and colleagues were the first to demonstrate the extra-lenticular expression of a crystallin [11]. Further studies revealed the presence of αB-crystallin in numerous tissues and its increased accumulation in neurological disorders [6,12-14]. Later, it was demonstrated that αA and αB-crystallins have chaperone-like activity [15], are phosphorylated in vivo and possess autokinase activity [16,17], interact with cytoskeleton [18], and protect cells from thermal and metabolic stress [19]. Furthermore, their ability to prevent apoptosis by inhibiting caspases indicates that αA and αB-crystallins have more general physiological functions in non-lens tissues [20].

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Retinal expression of crystallin genes was initially documented in the chicken [21]. Low levels of α-crystallin were also detected in frog retinal photoreceptors (in post-golgi membranes) and suggested to play a role in rhodopsin trafficking [22]. Later, crystallin expression was demonstrated in the retina of several species [23-29]. Furthermore, expression levels of crystallins (αA, αB, several members of the β-crystallin family and γS-crystallin) were shown to be modulated under stress conditions [30-32]. Increased expression of several crystallin genes in light damaged photoreceptors and the decreased expression of αA-crystallin in the retinal dystrophic rat suggested a possible role of crystallins in protecting the photoreceptors from light damage [30,31]. An intriguing recent finding is the identification of crystallins as components of retinal drusen isolated from human donor retinas of aging individuals and of patients with age-related macular degeneration [30]. Although detected in the retina and other tissues, functions of β, and γ-crystallins remain unclear [28,29]. It has not been determined whether α, β, and γ-crystallins have a specific role in the retina or represent an adventitious form of expression, perhaps rudiments of early interactions between the developing lens and the optic vesicle [29].

Mice lacking αA ($\alpha A^{-/-}$) or αB ($\alpha B^{-/-}$) have provided considerable insights into the functional roles of these proteins [33,34]. For example, αA may be necessary for maintaining the solubility of other crystallins in the lens. More broadly, α-crystallins are suggested to enhance cell survival and genomic integrity in lens epithelial cells [35-38]. The deletion of αA or αB gene appears to have distinct effects [35,37]. The absence of αA increases cell death in vivo during mitotic phase [38]. In contrast, deletion of αB-crystallin produced cells that have a greater tendency to hyper-proliferate in culture, indicating a possible role of αB in maintaining genomic stability [37].

Spatial profiling of crystallin transcripts and protein expression can provide an important tool to decipher the function of these proteins in the retina [39,40]. Recent efforts utilizing functional genomics have identified the involvement of crystallin gene families during aging and disease conditions [40,41]. We therefore performed a comprehensive analysis of crystallin gene and protein expression in the adult mouse retina. Here we report extensive expression analysis of crystallins using quantitative RT-PCR, followed by immunoblotting and immunocytochemical analysis using several well-defined crystallin antibodies.

METHODS

Isolation of retinas: Retina samples for RNA and protein analysis were dissected from adult C57BL/6 mice, snap frozen and stored at -80 °C, in accordance with the Institutional policies on the care and use of laboratory animals in research. In addition, retinas were isolated from adult 129Sv strain of mice. Mice lacking αA-crystallin (αA-/-) or αB-crystallin (αB-/-) on a 129Sv background were generously provided by Dr. Eric Wawrousek (National Eye Institute), and were used as negative controls for expression of these proteins. Twelve to thirteen week old mice were used in these studies. Retinas were dissected between 3 and 4 PM (subjective midday).

Quantitative RT-PCR (qRT-PCR): Gene specific primers (Table 1) were derived from murine genomic DNA sequences for crystallin genes (Ensembl database). For each gene, a primer set was designed to amplify a 200-400 bp product from the cDNA. Primer pairs spanned at least one intron so that the amplification due to genomic DNA contamination could be detected as it would produce a larger amplicon of >800 bp. The qRT-PCR analysis was performed using iCycler optical detection system (Bio-Rad) to measure fluoresence produced by SYBR Green I dye (Molecular Probes, Eugene, OR) intercalating into PCR product. Pairs of retina from seven 10 week old wild-type mice were dissected; four of these were pooled, and the other three were processed separately. Total RNA was prepared using the Trizol reagent (Invitrogen), and the cDNA template was generated using the Superscript system (Invitrogen). PCR reactions for each gene were performed in triplicate on each cDNA template along with triplicate reactions of a neuronal housekeeping gene, Hypoxanthine Guanine Phosphoribosyl Transferase (Hprt). The integrity of PCR reaction was verified by melt-curve analysis and agarose gel electrophoresis. The threshold cycle (Ct) difference between Hprt and each crystallin gene was calculated. Each crystallin gene was tested in triplicate on a given RNA sample. On the same 96 well plate, Hprt was also tested in triplicate on the same RNA sample. Each set of triplicates yielded three Ct values. These Ct values were averaged and the difference between the Hprt Ct (Avg) and Crystallin gene Ct (Avg) was calculated (Ct-diff). Each crystallin gene (and Hprt controls) were tested using four RNA samples to determine the Ct-diff. Three of these samples were total RNA isolated from pairs of retinas from different mice and a fourth sample consisted of total RNA isolated from a pool of 8 mouse retinas. The four resultant Ct-diff values were averaged in order to calculate the reported fold change from Hprt. Each difference of 1 cycle corresponds to a 2-fold change in expression between Hprt and a particular crystallin gene assuming 100% reaction efficiency.

Immunofluorescence: Cryosections (10 μm) of adult mouse eye were probed with antibodies against bovine αA, αB, the βH-crystallin fraction and the total γ-crystallin fraction. The distribution of these proteins was analyzed by immunofluorescence and confocal microscopy. For αA-crystallin, a 1:20 dilution of a monoclonal antibody against bovine αA was used (kindly provided by Dr. Paul Fitzgerald). For αB, a 1:200 dilution of a polyclonal antibody raised against bovine αB (Novocastra Laboratories) was used. Both primary antibodies showed high specificity and gave low background in immunocytochemistry with $\alpha A^{-/-}$ and $\alpha B^{-/-}$ mouse lens slices, respectively [35,37]. A monoclonal antibody raised against the bovine βH-crystallin was used at 1: 200 dilution; this antibody recognizes the βB2 protein. A polyclonal antibody that was raised against the bovine γ-crystallin fraction recognized γB, γC and γD-crystallins [42] and was used at 1:200 dilution. In each case, frozen eye sections were fixed for 10 min with 95% ethanol, hydrated and then blocked with 20% normal donkey serum in PBS containing 0.1% Triton X-100, for 30 min. They were then incubated overnight at 4 °C with the pri-

mary antibody. After three 10 min washes in PBS, the sections were blocked with 20% normal goat serum for 20 min, and incubated with Alexa^{568}-conjugated goat anti-mouse or goat anti-rabbit IgG used as the secondary antibody at 1:300 dilution.

Table 1. Gene specific primers for crystallins used in RT-PCR

Gene	Ensembl Transcript ID	Forward Primer	Reverse Primer
Cryaa	ENSMUST00000014690	ACAACGAGAGGCAGGATGAC	AGGGGACAACCAAGGTGAG
Cryaa-Ins	ENSMUST00000019192	TAATGCACCAACCACATGCT	ACATTGGAAGGCAGACGGTA
Cryab	ENSMUST00000034562	GCGGTGAGCTGGGATAATAA	GCTTCACGTCCAGATTCACA
Crya-nov1	ENSMUST00000044048	CGTGCTTCAGCTCCTTTACC	GACAGGACACCCTCAGGAGA
Cryba1	ENSMUST00000000740	AACTTCCAGGGCAAGAGGAT	AGATGGGTCGGAAGGACAT
Cryba2	ENSMUST00000006721	GACACTGTTTGAGGGGGAAA	CCTGTGTGCCAAAGTCACTG
Cryba3	ENSMUST00000060665	TCCAACCACCAAGATGGCTCAG	ATCACAGATTTCCCACTGGCGTCC
Cryba4	ENSMUST00000031285	GGTGCGATCTCTCAAAGTCC	TGCAGAGAGGGATAGTCATCG
Crybb1	ENSMUST00000031286	CCTCTGGGTTTATGGCTTCT	AGCCCTCTTGGTGCCACT
Crybb2	ENSMUST00000031295	GGCTACGAGCAGGCTAATTG	CCTTGTAATCCCCCTTCTCC
Crybb3	ENSMUST00000031297	GAGGCAGAAGTATCCCCAGA	GGAGGGACAGGAGAATGTCA
Cryga	ENSMUST00000027088	CTCCTGCCGTTCCATTCCAT	GTCGTGGTAGCGCCTGTAGT
Crygc	ENSMUST00000027089	TGCTGCCTCATCCCCCAACA	TCGCCTAAAAGAGCCAACTT
Crygd	ENSMUST00000027091	TGCCGCCTCATCCCCCACGCCG	GTCGTGGTAGCGCCTGTACT
Cryge	ENSMUST00000045028	ACCCTGACTACCAGCAGTGG	GTCCAGATGGAGAAAATGGT
Crygf	ENSMUST00000027082	GTGGCTGCTGGATGCTCTAT	GCCTATACTCCCCTGGCCTC
Crygs	ENSMUST00000040592	TGCGGGAATCAACCTTTGC	GCCTTCTACCACCTTACAGGAATG
Crym	ENSMUST00000033198	CCTGAAGGAGTCAGGAGACG	GCCATCACCCCTTAACAGAA
Cryz	ENSMUST00000029850	GCGACAGGGTCTTCTGCTAC	TGTGCCCAAAACCTTTAAGC
CryL	ENSMUST00000022517	AGCTCTTGTCTGCTGCCTTC	TAGTCTCCAAGGGTCCGATG
Hprt	ENSMUST00000026723	CAAACTTTGCTTTCCCTGGT	CAAGGGCATATCCAACAACA

Primer pairs for each murine crystallin gene were designed using the Primer3 software (version 0.2c) hosted at the Whitehead Institute and based upon sequence information found in the Ensembl database.

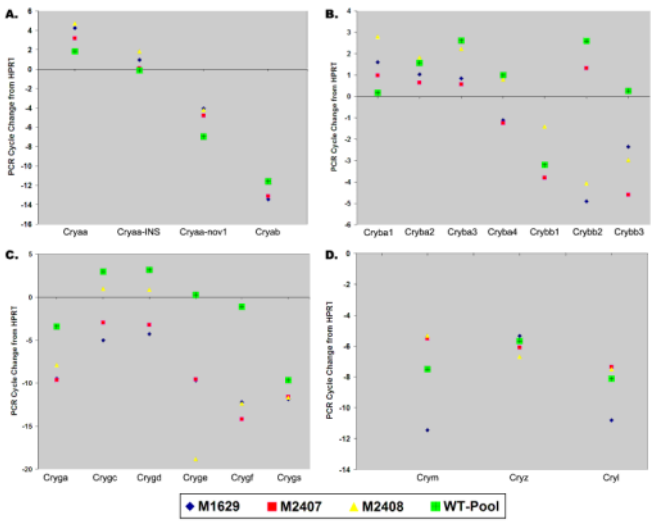

Figure 1. Assessment of retinal crystallin gene copy number by quantitative RT-PCR. Four different mouse retinal samples were examined to determine the mean PCR cycle change from Hprt for each crystallin gene. Three pairs of retinas isolated from different mice (M1629, M2407, M2408) and a fourth sample consisting of 8 mouse retinas (WT-Pool) were analyzed. Total RNA was extracted from these samples and served as a template for reverse transcription. Primer pairs were designed to flank introns so PCR amplicons produced from a genomic DNA template could be detected. A cycle change of (+1) from Hprt represents a hypothetical two-fold increase in the mRNA abundance for that gene compared to Hprt mRNA expression levels. Every sequential increase of (+1) would represent a further two-fold increase in abundance of that specific transcript assuming 100% reaction efficiency in the RT-PCR. Data for α, β, γ, and other (μ, ζ, λ) crystallin gene members is are shown in panels **A**, **B**, **C**, and **D**, respectively.

To demonstrate antibody-specificity, we examined retina sections from $\alpha A^{-/-}$ and $\alpha B^{-/-}$ mouse eyes. In addition, we used retina sections with no primary antibody added and with primary antibodies after antigen adsorption, as negative controls.

To visualize the nuclei, retinal sections were stained with TOTO-1 (Molecular Probes). The sections were viewed using Zeiss LSM 410 confocal microscope, equipped with an Argon-Krypton laser [35,37].

Immunoblot analysis.: Retinas, harvested from 12.5 week old mice, were lysed in 100 μl of lysis buffer (containing 40 mM Tris.Cl pH 7.5, 300 mM NaCl, 1% Triton X-100, 0.2% deoxycholate, 2 mM PMSF, 0.2% SDS and protease inhibitor cocktail [Sigma]) for 30 min at 4 °C. After sonication, the lysates were centrifuged for 30 min, and the supernatant was mixed with 30 μl of the SDS-PAGE sample buffer (5X) containing 0.02 M Tris.Cl pH 6.8, 4% β-mercaptoethanol, 4 mg/ml bromophenol blue, 5% SDS and 60% glycerol. A 15 μl aliquot containing approximately 20 μg of detergent-soluble retina protein was analyzed by SDS-PAGE using 15% gels [35]. Proteins were transferred to PVDF membranes and probed with primary antibodies to αA, αB, βH and γ-crystallins, as described above. The secondary antibodies were HRP-labeled anti-mouse or HRP-labeled anti-rabbit IgGs. Blots were incubated with Luminol reagent (Santa Cruz Biotechnology) and exposed to Kodak film to visualize the protein bands. Equal protein loading of retina lysates from different animals was confirmed by reprobing blots with a monoclonal antibody to β-tubulin as a housekeeping gene. In these analyses, α, β, and γ-crystallin fractions isolated by size-exclusion chromatography from mouse, bovine or human lenses, or mouse lens epithelial cell lysates derived from 5x10^4 cells were used as controls. Protein concentration of the isolated fractions of α, β, and γ-crystallin was determined based on

Table 2. Retinal crystallin transcript levels relative to Hprt

Gene	Mean difference of cycle threshold from Hprt (±SEM)	Fold difference from Hprt (Range)
Alpha-A-Crystallin	2.76 ± 0.45	5.4 / 8.1
Alpha-A-INS-Crystallin	0.33 ± 0.27	0.1 / 2.5
Alpha-B-Crystallin	-12.09 ± 0.29	-4355.3 / -4352.8
Alpha-A-Crystallin-nov1	-6.15 ± 0.44	-72.2 / -69.5
Beta-A1-Crystallin	0.86 ± 0.36	0.5 / 3.1
Beta-A2-Crystallin	1.40 ± 0.14	1.5 / 3.7
Beta-A3-Crystallin	2.01 ± 0.32	2.8 / 5.3
Beta-A4-Crystallin	0.35 ± 0.37	0 / 2.6
Beta-B1-Crystallin	-3.03 ± 0.26	-9.4 / -7
Beta-B2-Crystallin	0.39 ± 1.18	-1 / 3.6
Beta-B3-Crystallin	-1.26 ± 0.71	-4 / -0.8
Gamma-A-Crystallin	-5.80 ± 1.07	-58 / -53.8
Gamma-C-Crystallin	0.71 ± 1.17	-0.6 / 3.9
Gamma-D-Crystallin	0.86 ± 1.15	-0.4 / 4.0
Gamma-E-Crystallin	-5.28 ± 2.65	-45.1 / -32.5
Gamma-F-Crystallin	-6.17 ± 2.22	-76.5 / -67.2
Gamma-S-Crystallin	-10.52 ± 0.39	-1470.7 / -1468
Mu-Crystallin	-7.47 ± 0.70	-178.8 / -175.5
Zeta-Crystallin	-5.83 ± 0.15	-58 / 55.8
Lambda-Crystallin	-8.30 ± 0.40	-317.4 / -314.7

Quantitative analysis of crystallin transcripts in the retina by qRT-PCR. Hprt expression was used to normalize the expression levels. For each crystallin transcript, triplicate reactions were performed on four different total RNA samples. The hypothetical value of $2^{\text{cycle threshold difference}}$ (100% reaction efficiency assumed) was used in calculating the fold difference from Hprt.

aromatic amino acid compositions [43,44]. Quantitative immunoblot analysis was carried out by SDS-PAGE of varying concentrations of lens α, β, and γ-crystallins using well-characterized antibodies. These analyses indicated that αA-crystallin had higher expression in retina than αB-crystallin. Detergent-insoluble fractions of retinal lysates were also analyzed.

RESULTS

Expression of crystallin transcripts in the mouse retina: While generating gene expression profiles by custom eye-gene arrays [40,41] or Affymetrix GeneChips (Yoshida and Swaroop, unpublished data), we discovered an abundance of transcription of crystallin family genes in the retina. α, β, and γ-Crystallins are the major components of vertebrate lenses and are related to molecular chaperones and bacterial stress proteins [4,6,8]. Though expression of some of the crystallins has been shown in the retina, a comprehensive gene and protein expression profile of crystallins is not yet reported. We therefore employed real-time RT-PCR (quantitative RT-PCR; qRT-PCR) to examine the expression of all known crystallin genes in the retina.

We amplified the transcripts for 20 different crystallin family genes, which include a previously unreported, novel member of the αA-crystallin family termed αA-nov1 (Ensembl Transcript ID ENSMUST00000044048), three known genes of the α-crystallin family, thirteen genes of the β/γ-crystallin superfamily, and the taxon-specific μ, λand ζ-crystallin genes. Sequence and transcript information for each crystallin gene was obtained by querying the Ensembl Mouse Genome server

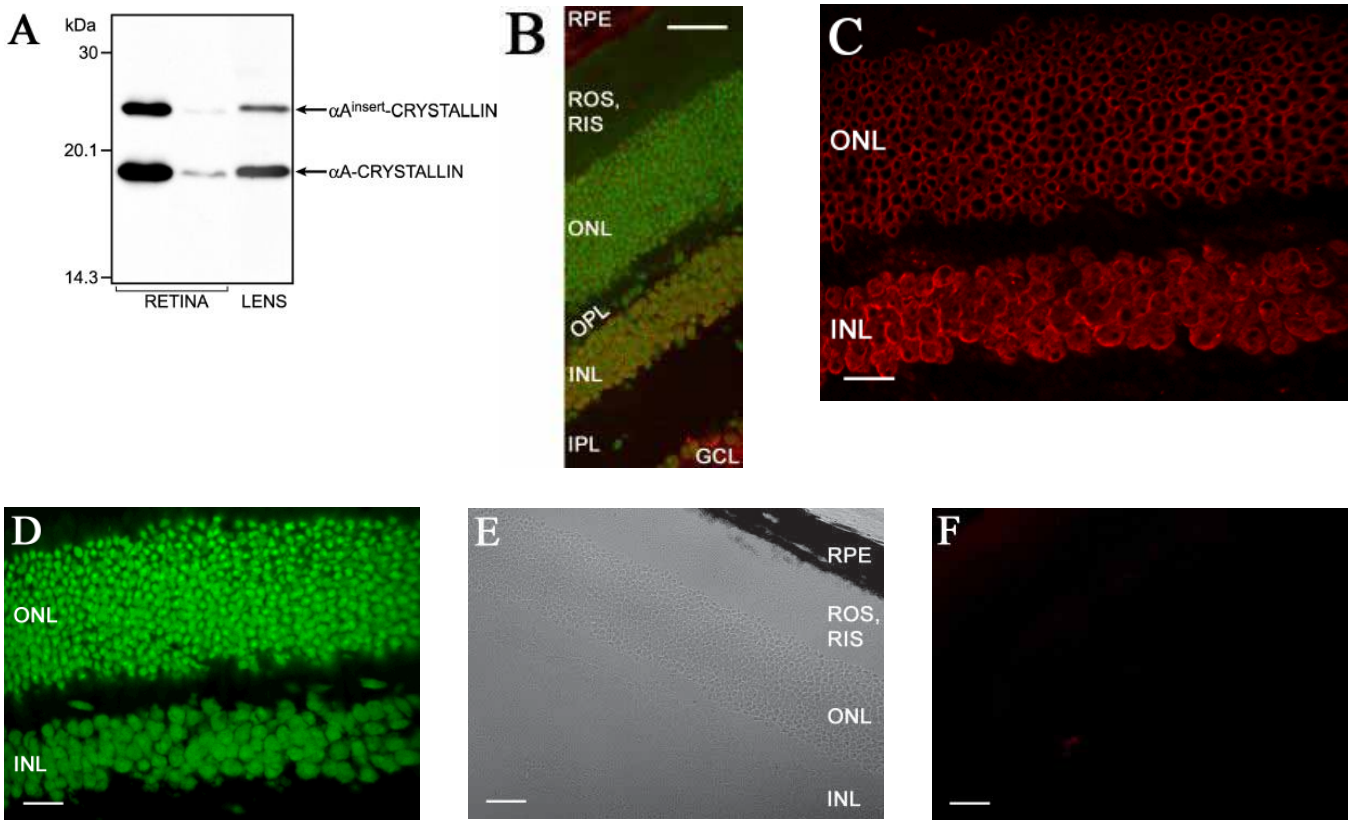

Figure 2. Expression of αA-crystallin in mouse retinas. Expression of αA-crystallin in adult mouse retinas by immunoblotting and immunofluorescence. Immunoblot analysis. (**A**) Cell lysates were prepared from 12.5 week old retinas and analyzed by SDS-PAGE and immunoblotting. A 20 μg aliquot of the retinal protein was applied to the gel. The monoclonal antibody against bovine αA-crystallin was used. Lanes are: left and middle, mouse retinas of two different animals; right, mouse lens epithelial cells ($5x10^4$, corresponding to 0.3 μg of αA-crystallin). Note the significant variability in expression of αA-crystallin in retina derived from two animals of the same litter. Note also that the αA- and αA-insert proteins from the retinas had the same mobility as the proteins from lens epithelial cells. Immunofluorescence. (**B**) αA-crystallin (red) was localized using an antibody to αA-crystallin, and nuclei (green) were stained with TOTO-1. Cellular morphology was visualized with differential interference contrast (DIC). Note that αA-crystallin was distributed in the ganglion cell layer, inner photoreceptor layer, and outer nuclear layer (**B**). A higher magnification of αA-crystallin distribution in the inner and outer nuclear layers of the retina is shown in (**C**). Note that αA-crystallin distribution was restricted to the membranes of the outer nuclear layers, but it is also distributed in the structures within the nucleus of the inner nuclear layer. A high magnification image of TOTO-1 immunofluorescence of the nuclei shown in (**C**) is shown in (**D**). A DIC image of an $\alpha A^{-/-}$ retina (negative control) is shown in (**E**). No αA-crystallin immunofluorescence was detectable in the $\alpha A^{-/-}$ mouse retina. Bar represents 13 μm (**B**, **E**, **F**); 5 μm (**C**,**D**). RPE, retinal pigment epithelium; ROS, rod outer segments; RIS, rod inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

hosted at The Sanger Institute. Since much homology is shared among members of the various crystallin families, PCR primers were designed in non-homologous regions and reaction fidelity was verified by DNA sequencing.

Figure 1 shows the cycle threshold difference compared to Hprt obtained with gene specific primers (Table 1) for various crystallin genes, depicting the relative expression of the transcript for each crystallin. A summary of the expression of transcripts for various crystallins, relative to the enzyme Hprt (used here as a reference) is provided in Table 2. The relative amounts of αA and αB-crystallin transcripts can be determined by the data presented in Table 2. For example, αA-crystallin has a cycle threshold difference from Hprt of (+2.76) and αB crystallin has a cycle threshold difference from Hprt of (-12.09). Hence, αA-crystallin appears +14.85 cycles (+2.76 to -12.09) before αB-crystallin, corresponding to a 29,532 ($=2^{14.85}$) fold higher expression of the αA-crystallin transcript than αB-crystallin in the retina. This calculation could be applied to determine relative transcript amounts for any of the crystallin genes reported in this table, assuming 100% reaction efficiency.

Expression of crystallin proteins in the mouse retina: **αA-crystallin:** In the lens, αA-crystallin represents the major protein of the α-crystallin gene family and is known to act as a molecular chaperone [15]. $\alpha A^{-/-}$ mice have reduced lens size and $\alpha A^{-/-}$ lens epithelial cells demonstrate slower growth [33,35]. Since αA-crystallin transcripts were detected by microarray analysis and qRT-PCR, we tested for the presence

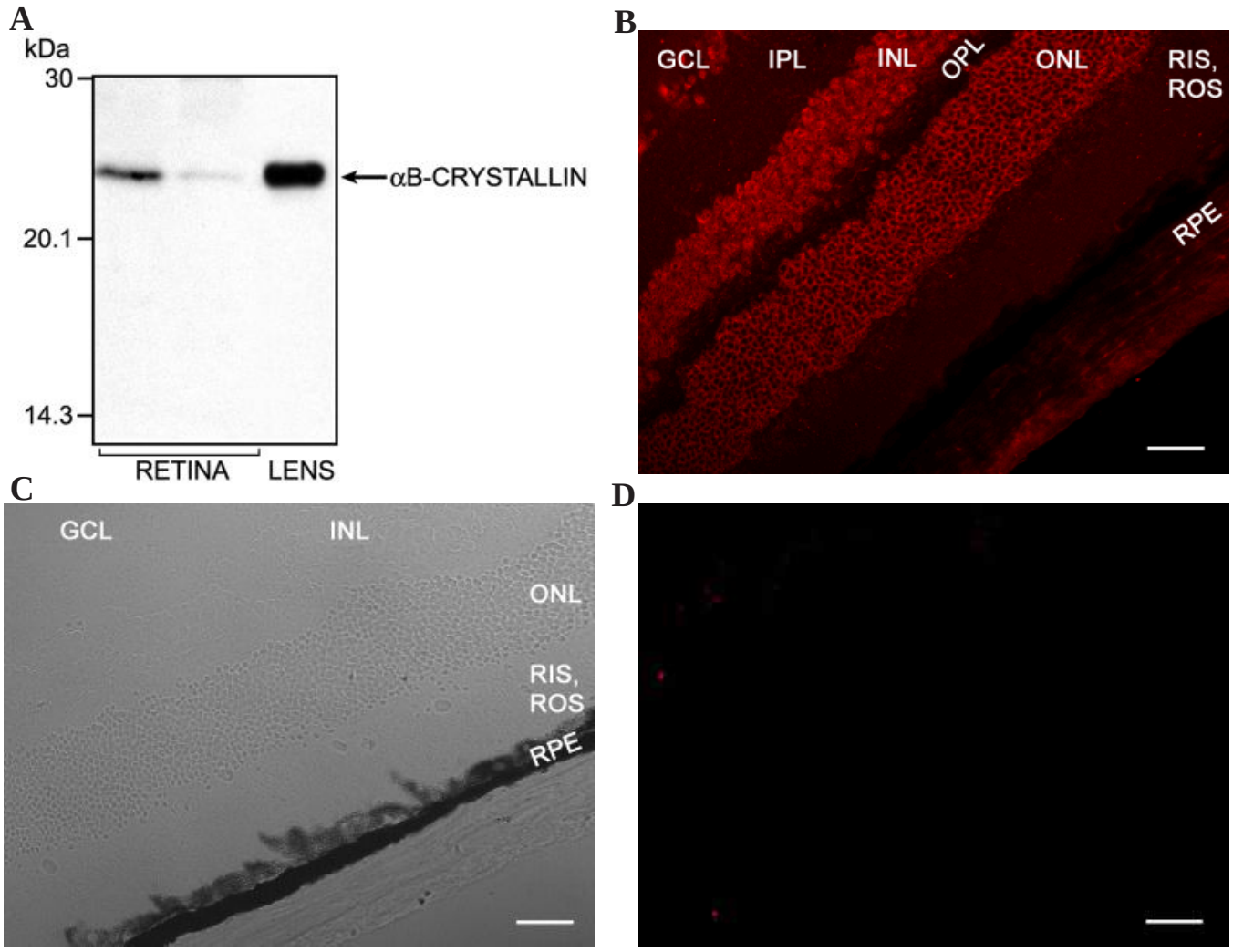

Figure 3. Expression of αB-crystallin in mouse retinas. Expression of αB-crystallin in adult mouse retinas by immunoblotting and immunofluorescence. Immunoblot analysis. (**A**) Cell lysates were prepared from 12.5 week old retinas and analyzed by SDS-PAGE and immunoblotting. A 20 μg aliquot of the retinal protein was applied to the gel. A polyclonal antibody against bovine αB-crystallin was used. Lanes are: left and middle, mouse retinas of two different animals; right, human lens αB-crystallin (0.1 μg). Note the significant variability of expression of αB-crystallin in retina derived from two animals of the same litter. Immunofluorescence. (**B**) αB-crystallin (red) was localized using an antibody to αB-crystallin. Cellular morphology was visualized with differential interference contrast. Note that αB-crystallin was distributed in the ganglion cell layer, inner photoreceptor layer, and outer nuclear layer (**B**). Note also the punctate staining of αB in the photoreceptor inner segments. A DIC image of an $\alpha B^{-/-}$ retina (negative control) is shown in (**C**). In the $\alpha B^{-/-}$ retina, there was no detectable αB-crystallin immunofluorescence (**D**). Bar represents 13 μm (**B**-**D**).

of αA protein in the adult mouse retinas by immunoblotting and immunolocalization studies (Figure 2). In retinal cell lysates, a prominent band at the expected molecular weight of approximately 20 kDa was detected (Figure 2A). This band also was observed in the lens epithelial cell cultures derived from adult mice. A second, higher molecular weight band at approximately 24 kDa in retinal lysates probably represents the αA-insert protein, a product of alternative splicing of the mRNA [1]. The αA and αA-insert proteins from retinal cell lysates had the same mobility as the proteins detected in mouse lens epithelial cells. There was no cross-reactivity of the antibody with any other protein on the immunoblots. Significant variation between αA-crystallin amounts was observed between retinas from different animals. For example, quantitative immunoblot analysis of two different retinas (Figure 2A) showed a difference of ten fold. For these two samples, the amount of retina protein loaded was the same. Re-probing the immunoblot with an antibody to β-tubulin as an internal loading control confirmed equal protein loading for the two retina samples (data not shown). The variations in expression were evident for of all crystallins examined by immunoblot analysis. This variability in expression was not due to a redistribution of crystallin to the detergent-insoluble cytoskeletal/membrane fraction of the retina, since these fractions showed a parallel variability (data not shown). The expression of αA-crystallin in retinas was lower than that in the lens, where αA and αB-crystallin together account for nearly 40% of the total water-soluble protein [15].

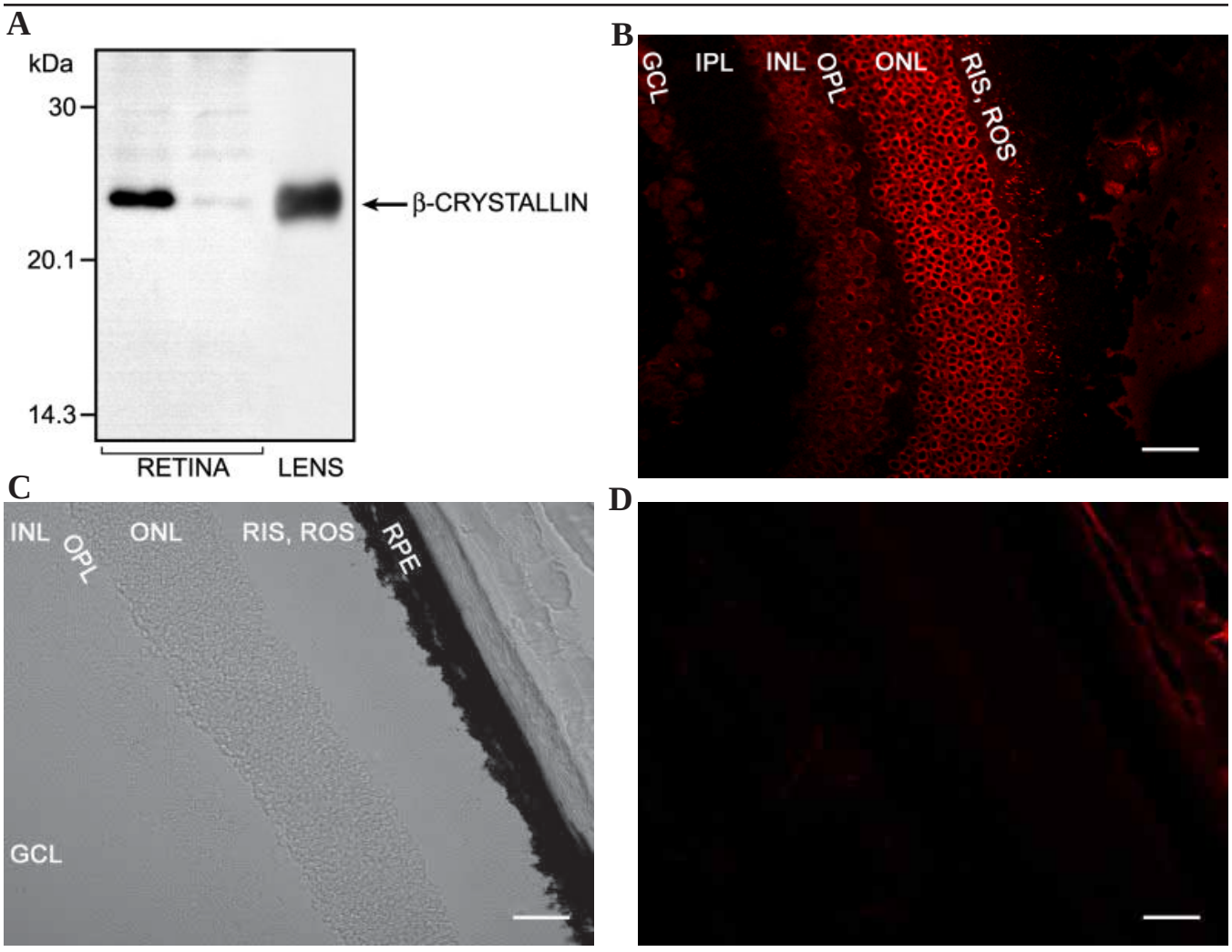

Figure 4. Expression of β-crystallin in mouse retinas. Expression of β-crystallin in adult mouse retinas by immunoblotting and immunofluorescence. Immunoblot analysis. (**A**) Cell lysates were prepared from 12.5 week old retinas and analyzed by SDS-PAGE and immunoblotting. A 20 μg aliquot of the retinal protein was applied to the gel. A monoclonal antibody against the bovine βH-crystallin fraction was used. Lanes are: left and middle, mouse retinas of two different animals; right, bovine lens βH-crystallin (1 μg). Note the significant variability of expression of β-crystallin in retina derived from two animals of the same litter. Immunofluorescence. (**B**) β-crystallin (red) was localized using a monoclonal antibody against bovine βH-crystallin fraction. Note that β-crystallin was distributed in the ganglion cell layer, inner photoreceptor layer, and outer nuclear layer. Note also the prominent punctate staining of β-crystallin in the photoreceptor inner segments. Cellular morphology was visualized with differential interference contrast. A DIC image of a normal retina is shown in (**C**). Pre-adsorption of the primary antibody with bovine βH-crystallin protein (negative control) showed no detectable β-crystallin immunofluorescence (**D**) in the retina shown in (**C**). Bar represents 13 μm (**B**-**D**).

The distribution of αA-crystallin in different cell layers of the retina was visualized by immunofluorescence and confocal microscopy. Dual staining with the αA antibody (shown in red) and the DNA stain TOTO-1 (green) was performed. As shown in Figure 2B, αA was distributed in the ganglion cell layer nuclei, and the inner and outer photoreceptor nuclear layers. Interestingly, αA was undetectable in photoreceptor inner and outer segments. Higher magnification images of the αA immunofluorescence (Figure 2C) and nuclei (Figure 2D) showed that the distribution of αA in the inner and outer nuclear layers was distinctive. In the inner nuclear layer, it appeared to label membranes and other cytoskeletal structures, whereas in the outer nuclear layer, αA distribution was restricted to the nuclear membranes. αA immunofluorescence was undetectable in the $\alpha A^{-/-}$ retinas (Figure 2E,F), confirming the specificity of the antibody.

αB-crystallin: In the lens, α-crystallin exists as a high molecular weight complex of two polypeptides, αA and αB in 3:1 stoichiometry [15]. Because αA can influence the distribution of αB in the lens [33], and since αB transcripts were also detected by microarray analysis and qRT-PCR, we tested for the presence of αB in mouse retinas. αB was detected as a single immunoreactive band in retina lysates, and migrated at the same position as αB isolated from the lens (Figure 3A). Variability of expression of αB-crystallin was observed among different retinas examined. Figure 3A shows an immunoblot analysis of retinal cell lysates from two different animals having a five-fold difference in expression. The amount of retinal protein applied to the gel was the same. Quantitative immunoblot analysis showed that the amount of αB-crystallin was 15 to 30 fold lower than that of αA-crystallin.

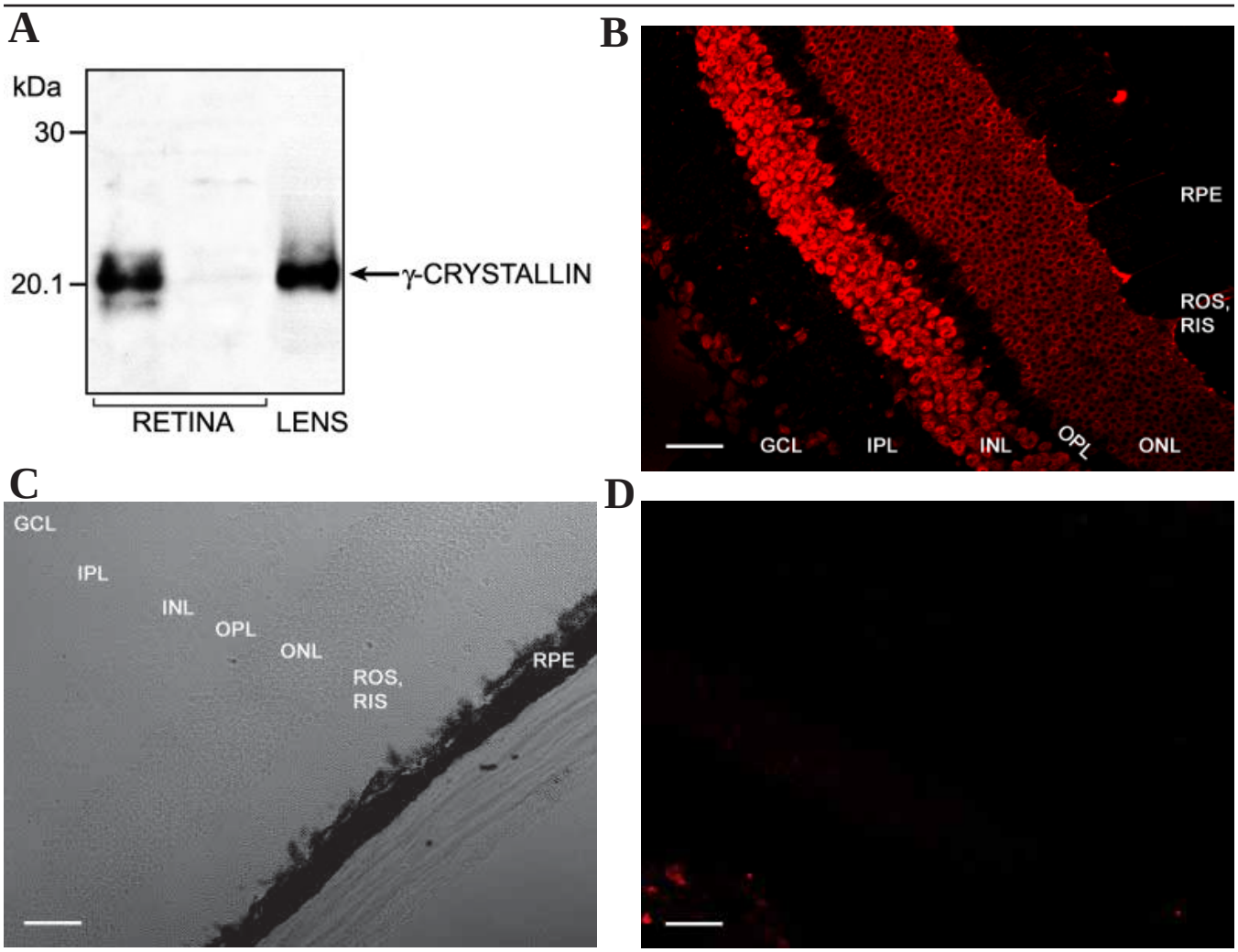

Figure 5. Expression of γ-crystallin in mouse retinas. Expression of γ-crystallin in adult mouse retinas by immunoblotting and immunofluorescence. Immunoblot analysis. (**A**) Cell lysates were prepared from 12.5 week old retinas and analyzed by SDS-PAGE and immunoblotting. A polyclonal antibody against bovine γ-crystallin fraction was used. Lanes are: left and middle, mouse retina; right, bovine lens γ-crystallin (0.7 μg). Note the significant variability of expression of γ-crystallin in retina derived from two animals of the same litter. Immunofluorescence (**B**) γ-crystallin (red) was localized using a polyclonal antibody against bovine γ-crystallin fraction. Note that γ-crystallin was distributed in the ganglion cell layer, inner photoreceptor layer, and outer nuclear layer. Cellular morphology was visualized with differential interference contrast. A DIC image of a normal retina is shown in (**C**). Pre-adsorption of the primary antibody with bovine γ-crystallin protein (negative control) showed no detectable γ-crystallin immunofluorescence (**D**) in the retina shown in (**C**). Bar represents 13 μm (**B**-**D**).

The distribution of αB-crystallin in different cell layers of the retina is shown in Figure 3B. Interestingly, αB was also distributed in the same cell layers of the mouse retina as αA, but in addition to its detection in the ganglion cell nuclei, and the inner and outer nuclear layers of the photoreceptor cells, some αB was also detected in the inner segments of photoreceptors. αB immunofluorescence was undetectable in retinas of αB-/- mice, confirming the specificity of the antibody (Figure 3C,D).

β-crystallins: These proteins exist as oligomeric complexes of several polypeptides. Two major fractions, βH and βL, can be isolated from bovine lenses by size exclusion gel chromatography, whereas human lenses contain β1, β2, and β3 fractions [45,46]. The β-crystallin fractions are complex and contain a number of peptides of variable stoichiometry. Immunoblotting of cell lysates from adult mouse retinas showed a single band at approximately 25 kDa co-migrating with the protein recognized by a monoclonal antibody to bovine lens βH-crystallin (Figure 4A). As in the case of αA and αB-crystallin, immunoblot analysis of retinas from different animals showed a wide range of β-crystallin expression. Figure 4A shows that retinas isolated from two different animals had a ten-fold difference in expression of β-crystallin. The amount of retina protein applied to the immunoblot was the same for these two samples.

Immunofluorescence analysis showed that like αB, the β-crystallin proteins were detected in all nuclear layers of the retina (Figure 4B), and interestingly, they also were prominently detected in the inner segments of the photoreceptor cells. No β-crystallin immunofluorescence was detected in the retina after pre-adsorption of the primary antibody with bovine β-crystallin protein (Figure 4C,D).

γ-crystallins: These are monomeric proteins present in lens and belong to the β/γsuperfamily of vertebrate crystallins. Crystallographic analysis has demonstrated the presence of a two-domain structure with four Greek key motifs that are related to bacterial stress proteins [5,8]. The presence of the γ-crystallin family has been demonstrated in 10-20 day old mouse retinas [29]. In our work, the transcripts for these crystallins were detected by qRT-PCR analysis of adult mouse retinas. Using an antibody that recognizes γB, C, and D family members, we investigated the expression of these crystallins in the adult mouse retinas. Immunoblot analysis demonstrated the expression of three closely spaced immunoreactive bands A (Figure 5A). The bovine lens γ-crystallin fraction was used as a positive control, and co-migrated with the γ-crystallin from mouse retinas. The minor bands migrating above and below the purified γ-crystallin are most likely due to slight degradation and crosslinking of the retina sample. Variability of retina expression was also observed in the case of γ-crystallin from different animals, as shown in Figure 5A.

Immunofluorescence studies showed that γ-crystallin was distributed in all the nuclear layers of the retina (Figure 5B). No γ-crystallin immunofluorescence was detected in the retina after pre-adsorption of the primary antibody with bovine γ-crystallin protein (Figure 5C,D).

DISCUSSION

Crystallins, previously thought to be components of only the lens, have come under intense scrutiny because of their possible function as chaperones or stress-response proteins. While all vertebrate lenses contain representatives of α, β, and γ-crystallins, some species or select taxonomic groups also express entirely different proteins as lens crystallins, such as the μ, ζ, and λcrystallins [1-3]. Several reports have identified the expression of one or more of these crystallins in the retina [21-29], and suggested significant environmental effects on their expression, including circadian, diabetes, aging, and intense light [30,31,41,47,48]. To our knowledge, this is the first comprehensive report providing evidence for the presence of crystallin gene transcripts (including several previously unreported crystallin family genes that were identified by in silico mining of genomic sequences) in the adult mouse retinas. The transcript analysis suggests that some genes are highly expressed (e.g., αA-crystallin), whereas others are minor (e.g., γS- and λ-crystallin), and their significance is not known. Our studies also demonstrate the spatial distribution of various crystallins using specific antibodies.

Immunoblotting studies confirmed the expression of α, β, and γ-crystallins and immunofluorescence data indicated that each of the crystallin antibodies recognizes antigens localized in the nuclear layers of the retina. Only αB and β-crystallin were also detected in the photoreceptor inner segments. Though a few studies have reported αB-crystallin in photoreceptors and pigment epithelium [31] and γ-crystallin in photoreceptors and ganglion cell layers [29], our study demonstrates for the first time that the major vertebrate crystallins are primarily distributed in the nuclear layers of the adult mouse retina, and their pattern of expression is significantly different between the inner and outer nuclear layers.

The delineation of precise functions for α, β, and γ-crystallins in the retina requires further study. However, our results showing the localization of αA, αB, β, and γ-crystallins in the retinal nuclear layers is significant from two perspectives. First, αA and αB-crystallin prevent aggregation of partially denatured proteins, and have been suggested to play a role in cell proliferation and genomic stability [15,35,37,49,50]. A similar role is possible in the retina. Second, the ability of αA and αB-crystallins to prevent apoptosis [20,36], their in vivo phosphorylation [16], association with membranes [51,52] and cytoskeletal elements [18] and possible association with signal transduction pathways [17], and increased expression of αB in many diseased states [6], suggest the possibility that the crystallins may have a protective function in retinal cells. Since increased apoptosis of retinal photoreceptor and inner nuclear layers has been associated with retinal and macular degeneration [53], it would be interesting to determine the correlation between retinal degeneration and crystallin expression.

α-Crystallins are small heat shock proteins that act as molecular chaperones, and are distinguished from other chaperone families, such as HSP60 and 70, by their high capacity to bind non-native protein and their lack of ATP consumption,

making them an efficient defense mechanism under stress conditions [15,49]. A number of crystallin genes are activated by Pax-6, a conserved transcription factor for eye evolution [54]. αA and αB-crystallins are also differentially regulated at the transcriptional level and there is a marked specialization of the αA-crystallin gene promoter for expression in lens. The αB-crystallin gene has a very complex pattern of expression, in heart, lung, kidney, brain, eye and other tissues [6].

A noteworthy observation in the present work is the variations in the level of some of the crystallin RNA (Figure 1) and protein (Figure 2, Figure 3, Figure 4, and Figure 5) expression between individual animals of the same litter. Normal variations in the expression of various crystallin genes may be reflective of the stress level, metabolic status, and/or age of these animals. This variability in expression suggests that crystallin expression might be a sensitive indicator of metabolic status or stress response. We suggest that data on changes in crystallin expression should be interpreted cautiously and multiple data points should be obtained.

The role of α-crystallins as molecular chaperones and their ability to prevent the non-specific aggregation of denatured proteins have been well established. However, much less is known about the cellular functions of β/γ-crystallins, though they share structural characteristics and high intrinsic stability with microbial stress proteins [8]. Our studies are consistent with the hypothesis that αA and αB-crystallins, in addition to being molecular chaperones, may be involved in fundamental processes such as genomic stability. This report provides a basis for future studies on crystallins in the normal and diseased retinas. Changes in their expression and distribution with aging, retinal dystrophies, diabetes, and macular degeneration may possibly provide a direction for future therapies for blinding eye diseases.

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