Mechanism of Small Heat Shock Protein Function in Vivo

**A KNOCK-IN MOUSE MODEL DEMONSTRATES THAT THE R49C MUTATION IN \( \alpha A \)-CRYSTALLIN ENHANCES PROTEIN INSOLUBILITY AND CELL DEATH**

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\( \alpha A \)-crystallin (Cryaa/HSPB4) is a small heat shock protein and molecular chaperone that prevents nonspecific aggregation of denaturing proteins. Several point mutations in the \( \alpha A \)-crystallin gene cause congenital human cataracts by unknown mechanisms. We took a novel approach to investigate the molecular mechanism of cataract formation in vivo by creating gene knock-in mice expressing the arginine 49 to cysteine mutation (R49C) in \( \alpha A \)-crystallin (\( \alpha A \)-R49C). This mutation has been linked with autosomal dominant hereditary cataracts in a four-generation Caucasian family. Homologous recombination in embryonic stem cells was performed using a plasmid containing the \( \alpha A \) to T transition in exon 1 of the cryaa gene. \( \alpha A \)-R49C heterozygosity led to early cataracts characterized by nuclear opacity. Unexpectedly, \( \alpha A \)-R49C homozygosity led to small eye phenotype and severe cataracts at birth. Wild type littermates did not show these abnormalities. Lens fiber cells of \( \alpha A \)-R49C homozygous mice displayed an increase in cell death by apoptosis mediated by a 5-fold decrease in phosphorylated Bad, an anti-apoptotic protein, but an increase in Bcl-2 expression. However, proliferation measured by in vivo bromodeoxyuridine labeling did not decline. The \( \alpha A \)-R49C heterozygous and homozygous knock-in lenses demonstrated an increase in insoluble \( \alpha A \)-crystallin and \( \beta B \)-crystallin and a surprising increase in expression of cytoplasmic \( \gamma \)-crystallin, whereas no changes in \( \beta \)-crystallin were observed. Co-immunoprecipitation analysis showed increased interaction between \( \alpha A \)-crystallin and lens substrate proteins in the heterozygous knock-in lenses. To our knowledge this is the first knock-in mouse model for a crystallin mutation causing hereditary human cataract and establishes that \( \alpha A \)-R49C promotes protein insolubility and cell death in vivo.

\( \alpha A \)-crystallin is a member of the small heat shock protein family that includes 10 proteins in humans characterized by a conserved \( \alpha A \)-crystallin domain of ~90 amino acids in their C-terminal region (1). Point mutations in small heat shock protein genes are associated with pathological conditions such as cataracts and desmin-related myopathy (2–8). Mechanisms leading to these pathologies are currently under intensive investigation. The R116C and the R49C mutations in \( \alpha A \)-crystallin cause hereditary cataracts (2, 3). However, in vivo models that recapitulate the heterozygosity of human patients have not yet been developed.

The eye lens is an ideal model system to study small heat shock protein function because it is a simple cellular system with only two cell types, an anterior layer of cuboidal lens epithelial cells covering layers of uniquely elongated differentiated lens fiber cells. The development of the eye lens is a highly coordinated process involving intricate control of cell cycle regulation and differentiation (9, 10). During differentiation, lens fiber cells express a high abundance of crystallins, cytoplasmic proteins that are divided into two major families in vertebrate lenses, \( \alpha \)-crystallins and \( \beta B \)-crystallins. Essential for lens transparency, \( \alpha \)-crystallin is a large multicrystalline complex with an average aggregate molecular mass of ~500 kDa and is obtained as a complex of \( \alpha A \)-crystallin and \( \alpha B \)-crystallin when isolated from lens fiber cells (11, 12). \( \alpha A \)-crystallin constitutes nearly 20% of the soluble protein in newborn human lenses and acts as a molecular chaperone (13, 14). Gene knock-out mice have provided significant knowledge about \( \alpha A \)-crystallin functions and demonstrated that besides its role in refraction, it is an active polypeptide that has anti-apoptotic properties important for maintaining the survival of lens epithelial cells in vivo (15–17).

Among each of the autosomal dominant mutations that have been linked with hereditary cataracts, only the R49C mutation in \( \alpha A \)-crystallin has been found to lie outside the conserved C-terminal \( \alpha A \)-crystallin domain common to all small heat shock proteins (3). This mutation was linked with hereditary cataract in a four-generation Caucasian family. The C to T transition in codon 49 of exon 1 in the gene encoding \( \alpha A \)-crystallin results in the nonconservative substitution of arginine 49 to cysteine (R49C). No phenotypic and molecular characteristics of the cataract are available. The positive charge on arginine 49 in \( \alpha A \)-crystallin has been highly conserved during evolution, and it is essential for the in vitro chaperone activity of \( \alpha A \)-crystallin (18, 19). Transfection studies of the \( \alpha A \)-R49C protein in lens epithelial cell cultures have shown a 15-fold increase in basal cell death suggesting a gain of function phenotype of the mutant protein (3). Staurosporine-induced levels of cell death...
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increased 8-fold in the αA-R49C transfectants compared with wild type αA-crystallin. Although much can be learned from studying the mutant protein in vitro or in transgenic models, these studies have a number of limitations (3, 8, 20–23). Because how mutations affect protein interactions at low concentrations may have little relevance to how they associate in vivo at higher concentrations in the lens, an optimal design of a model must investigate the effect of the mutation in vivo. Point mutation gene knock-in mice are a powerful tool for dissecting gene function. Point mutation gene knock-in mice are those animals that have a point mutation in an endogenous gene that does not ablate the gene but merely changes its function (24). Gene knock-in mice have been used with great success to genetically dissect the role of specific genes; for example, the role played by specific lens connexin genes has been convincingly delineated with knock-in mice (25, 26), and they have a number of advantages. First, it is possible to analyze the effect of the mutation in every cell. Second, it allows a comparison of the advantages. First, it is possible to analyze the effect of the mutation in vivo at higher concentrations in the lens, an optimal design of a model must investigate the effect of the mutation in vivo. Point mutation gene knock-in mice are a powerful tool for dissecting gene function. Point mutation gene knock-in mice are those animals that have a point mutation in an endogenous gene that does not ablate the gene but merely changes its function (24).

In this study, we created the first gene knock-in mice to investigate the mechanism by which the R49C mutation in αA-crystallin produces cataract in vivo. We demonstrate that αA-R49C expression enhances protein insolubility and lens cell death in vivo leading to a small eye, small lens, and severe cataracts. Assessment of cell survival and signaling proteins suggests a link between in vivo cell death and dephosphorylation of the protein BAD, a central molecule involved in cell survival. Furthermore, the αA-R49C heterozygosity increased the interaction of key lens substrate proteins with the chaperone suggesting a higher level of unstable proteins in the mutant lenses. Together, these results extend our understanding of how αA-crystallin functions in vivo, and they support the idea that αA-crystallin is an active polypeptide critical to the development of a transparent lens phenotype.

EXPERIMENTAL PROCEDURES

Generation of Knock-in Mice—Knock-in mice were generated by removing the normal gene from one allele by homologous recombination in 129Sv1 male embryonic stem (ES)2 cells (SCC-10) to modify the αA-crystallin (cryaa) gene such that exon 1 contained the R49C mutation, whereas the second copy of the gene was wild type. Mouse genomic DNA clone derived from a 129Sv strain containing αA-crystallin gene was generously provided by Dr. Eric Wawrousek. The 2.9-kb 5′ arm was cloned into a cloning plasmid with the neomycin cassette. Exon 1 of the cryaa gene at codon 49 was mutated from C to T by site-directed mutagenesis (QuickChange kit, Stratagene). Next, the 4.8-kb 3′ arm of the cryaa gene was cloned into the plasmid (Fig. 1). The plasmid was electroporated into ES cells, and ES cell selection, colony picking, freezing, expansions, and cryopreservation of homologous recombinant clones expressing the R49C mutant αA-crystallin gene were performed at the Washington University ES Cell Core facility. Clones positive for neomycin were selected with G418, and 150 ES cell colonies were screened for correct gene targeting by Southern blot analysis. Four clones were found to be correctly targeted. The results presented here are from clone 85, which was positive for the mutation. The C to T mutation was verified by sequencing genomic DNA of the positive ES clones (Fig. 1B). Correct insertion of the knock-in allele was tested by probing 5′ and 3′ ends of the cryaa gene in the plasmid construct and with primers outside the cryaa gene. The neomycin-positive ES clones were analyzed by Southern blotting. ES cells positive for the mutation were karyotyped to check for normal chromosomes, injected into C57BL6 blastocytes, and implanted into pseudo-pregnant ICR females (27). Chimeric founders were mated with wild type C57BL6 mice, and their progeny that genotyped positive for germ line transmission were bred. First generation offspring that inherited the targeted allele with neomycin were subsequently backcrossed into C57BL/6J. The knock-in mice with the neomycin cassette were identified by PCR-based genotyping described in Fig. 1A (PCR product of 224 bp with pcr3 and pcr4 primers). Next, the heterozygous knock-in mice were bred with Cre EIIa transgenic mice in C57BL/6J genetic background to specifically delete the neomycin cassette (28, 29). All mice were further genotyped by Southern blot analysis of XhoI-digested DNA and hybridization with a 5′ end probe. Heterozygous offspring within each mating scheme were subsequently bred to yield homozygous mice. PCR genotypes of heterozygous and homozygous knock-in mice after deletion of the neomycin cassette are shown in Fig. 1D. Two independent lines of mice, R49CKI3 and R49CKI4, expressing the mutation in cryaa were bred. Because we started with a 129Sv mouse clone, which is known to express a deletion mutation in CP49, a lens fiber cell-specific protein, we backcrossed our αA-R49C knock-in mice with C57BL6 mice. These mice were further genotyped to exclude the presence of a deletion mutation in the gene for lens phakinin (CP49), which is characteristic of the 129 strain from which ES cells were derived. This analysis was done to verify that the cataract was not the result of the absence of wild type CP49 (30). Only those mice that were wild type for CP49 expression were analyzed. No mutant CP49 gene was detected in these mice (supplemental Fig. S1).

Mice were maintained at Washington University by the Division of Comparative Medicine, by trained veterinary staff. All protocols and animal procedures were approved by the Washington University Animal Studies Committee.

Genotyping—Genomic DNA was prepared from tail biopsy using the DNeasy spin column kit (Qiagen, Valencia, CA), according to the manufacturer’s instructions, and was quantified by absorbance at 260 nm. Mice were genotyped by PCR amplification (50 µL) of tail DNA (1 µg). To identify the knock-in construct containing neomycin cassette, the following primers were used: pcr3 (Cryaa Genotype 30-mer forward

2 The abbreviations used are: ES, embryonic stem; BrdUrd, 5-bromo-2′-deoxyuridine; WT, wild type; LC, liquid chromatography; nanoLC-FTMS/MS, linear quadrupole ion trap Fourier transform ion cyclotron resonance mass spectrometer; MS/MS, tandem mass spectrometry; PBS, phosphate-buffered saline; TUNEL, terminal dUTP nick-end labeling; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; LTQ, linear quadrupole ion trap; MIP, major intrinsic protein.

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The samples were then transferred to low bind autosampler vials, and the tubes were rinsed with 10 μl of 1% formic acid, 1% acetonitrile for nanoLC-FTMS.

NanoLC-FTMS Analysis—Mass spectrometry was performed using a linear quadrupole ion trap Fourier transform ion cyclotron resonance mass spectrometer (LTQ-FTMS, Thermoelectron, San Jose, CA) as described previously (31). The nanoliquid chromatograph (Eksigent NanoLC, Eksigent, Livermore, CA) was interfaced to the LTQ-FTMS with a PicoView nanocapillary source from New Objective (Woburn, MA). Sample injection was performed with an autosampler (Endurance, Spark, Plainsboro, NJ). The column was a C-18 PicoFrit (75 μm × 10 cm) (New Objective, Woburn, MA). The mobile phases were high pressure liquid chromatography grade water (Fisher) containing 1% formic acid (Sigma) (solvent A) and acetonitrile (Honeywell, Burdick & Jackson, Muskegon, MI) containing 1% formic acid (solvent B). The sample (5 μl) was loaded at 600 nl/min at 1% solvent B for 10 min. The flow was then decreased to 200 nl/min with isocratic elutions for 15 min that was followed by a linear increase in solvent B (0.3%/min) for 30 min. The LTQ FT (7 tesla) mass spectrometer was operated in the data-dependent mode. The survey scans (m/z = 350–2000) were acquired using Fourier transform ion cyclotron resonance with a resolution of ~100,000 at m/z = 421.75 with a target value of ~500,000. The 10 most intense ions from survey scans were isolated in the ion trap and analyzed after reaching a target value of ~10,000. The MS/MS isolation width was 2.5 Da, and the normalized collision energy was 35%. Electrospray ionization was accomplished with a spray voltage of 2.2 kV without sheath gas. The ion transfer tube temperature was 200°C.

MS Data Analysis—The MS and MS/MS data from the nanoLC-FTMSMS were collected in the profile mode. The “raw” files were processed using Mascot Distiller (Matrix Science, Oxford, UK) with the following settings. 1) For MS processing: 200 data points per Da; no aggregation method; maximum charge state = +8; minimum number of peaks = 1. 2) For MS/MS processing: 200 data points per Da; time domain aggregation method enabled; minimum number of peaks = 10; precursor charge and m/z, try to re-determine from the survey scan (tolerance = 2.5 Da); charge defaults = +2/+3; maximum charge state = +2. 3) For time domain parameters: minimum precursor mass = 700; maximum precursor mass = 16,000; precursor m/z tolerance for grouping = 0.1; maximum number of intermediate scans = 5; minimum number of scans in a group = 1. 4) For peak picking: maximum iterations = 500; correlation threshold = 0.90; minimum signal-to-noise = 3; minimum peak m/z = 50; maximum peak m/z = 100,000; minimum peak width = 0.001; maximum peak width = 2; and expected peak width = 0.01. The resulting Mascot generic files were exported to MASCOT, version 2.1.6. The tandem MS data were searched against the NCBI protein data base with the following constraints: MS tolerance = 10 ppm, MS/MS tolerance = 0.8 Da with fixed modifications of cysteine (carbamidomethylation) and methionine (oxidation) residues.

Slit Lamp Examination and Recording—Slit lamp biomicroscopy was used on nonanesthetized mice in a masked fashion. Pupils were dilated with a mixture of 10% phenylephrine hydrochloride and 1% tropicamide (Alcon, Fort Worth, TX). After 3
min, the animal was placed directly facing the slit lamp by holding the mouse gently by the scruff of the neck. The left eye of the animals was examined. The knock-in mice were examined at postnatal ages between 3 weeks (eye opening) and 36 weeks. To confirm lens opacity in newborn mice, newborn mouse pups were sacrificed and examined by slit lamp biomicroscopy. 

Assessment of Lens Opacity—Cataract formation was scored by slit lamp biomicroscopy according to a modified LOCS III method. For stage 0, clear lens; for stage 1, loss of normal appearance of anterior and posterior lens and prominence of y-suture line. Changes appear by 3 weeks in heterozygous lenses. For stage 2, discrete posterior changes were accompanied by light nuclear opacity. Changes were evident at 4 months in heterozygous lenses. For stage 3, there was a nearly mature cataract, involving approximately three-fourths of the lens with vacuoles and opacity. Changes were evident by 8 months in heterozygous and 3 weeks in homozygous lenses. For stage 4, a completely mature cataract involving the cortex with vacuoles was evident in heterozygous lenses not before 1 year and in homozygous lenses at 2 months.

Assessment of Proliferation—In vivo labeling with 5-bromo-2’-deoxyuridine (BrdUrd) was performed as described previously (17). Mice were injected with BrdUrd intraperitoneally (0.1 ml of a 10 mM solution of BrdUrd in sterile PBS) at 10 a.m. and were sacrificed 1 h later. Eyes were dissected and fixed, and mid-sagittal sections were labeled with a primary antibody to BrdUrd and a horseradish peroxidase-conjugated secondary antibody. Labeled nuclei were detected with an Olympus microscope. The total number of nuclei were measured by hematoxylin staining of the sections. The labeling index was determined by the ratio of the BrdUrd-positive nuclei and the total number of nuclei per section. For each sample, three sections per lens were analyzed, and labeling index was determined for six lenses per genotype.

Assessment of MIP (AQP0) Immunofluorescence—Lenses were embedded in paraffin, and 4-μm sections were stained with a polyclonal antibody to MIP (Alpha Diagnostics International), and an Alexa-568-conjugated secondary antibody, and visualized by confocal microscopy in a Zeiss 510 confocal microscope.

Assessment of Lens Protein Expression—Whole lenses were homogenized in PBS containing protease inhibitor mixture (Sigma) and centrifuged at 15,000 × g to separate soluble and insoluble fractions (20, 32). The protein concentration was measured by the Pierce BCA assay, and equal protein (15 μg) was loaded on the gel, unless otherwise noted. SDS-PAGE and immunoblot analysis was performed using previously described antibodies to αA-crystallin, αB-crystallin, total β-crystallin, and total γ-crystallin (16, 20, 33). MIP expression was detected using a polyclonal antibody to MIP (17) (Alpha Diagnostics International). Densitometric analysis was performed with a Storm 860 system (GE Healthcare).

Assessment of Disulfide Cross-linking—Whole lenses were homogenized in PBS without 10 mM dithiothreitol, and lens proteins were separated into soluble and insoluble fractions by centrifugation as described above. SDS-PAGE and immunoblot analysis with antibodies to αA-crystallin and γ-crystallin were performed.

Assessment of αA-crystallin Interaction with Lens Proteins by Immunoprecipitation—A co-immunoprecipitation assay was used to investigate proteins interacting with αA-crystallin in wild type lenses and to investigate the effect of the αA-R49C mutation on the interaction of αA-crystallin with lens proteins. Lenses were homogenized in PME buffer (80 PIPES, 1 mM MgCl₂, and 1 mM EGTA, pH 6.8) and centrifuged for 30 min at 10,000 × g. Supernatants were lysed for 30 min on ice with immune precipitation buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, and protease inhibitor mixture (Sigma) and centrifuged for 10 min at 10,000 × g. Supernatants were treated with a primary antibody to αA-crystallin and immunoprecipitated with protein A/G-agarose beads (Santa Cruz Biotechnology). A monoclonal antibody to αA-crystallin (1:20) was used. Immunoprecipitates were washed three times with a lysis buffer containing 20 mM Tris-HCl, 1% Triton X-100, 5 mM EDTA, 50 mM NaCl, and protease inhibitor mixture (Sigma), resuspended in SDS-PAGE sample buffer, and analyzed on 15% acrylamide gels as described previously (21). Immunoblot analysis with antibodies to αβ-crystallin, β-crystallin, γ-crystallin, and β-tubulin was performed.

Assessment of Cell Survival and Signaling Protein Expression—Freshly dissected lens epithelial and cortical fiber cells were analyzed by immunoblot analysis. Samples were extracted in 44.4 mM n-octyl β-D-glucopyranoside, 1% Triton X-100, 100 mM NaCl, 1 mM MgCl₂, 5 mM EDTA, and 10 mM imidazole containing 1 mM sodium vanadate, 0.2 mM H₂O₂, and protease inhibitor mixture (Sigma). Antibodies against Bcl-2 (Santa Cruz Biotechnology), phospho-Bad Ser-112, and phospho-Bad Ser-136 (BIOSOURCE), Akt and p-Akt (Cell Signaling Technology, Inc. Danvers, MA), p38 (Santa Cruz Biotechnology), and phospho-38 ERK1/2 and phosphorylated ERK1/2 (Promega, Madison, WI) were used for immunoblot analysis. Equal amounts of total cellular protein (15 μg) were separated on Tris-glycine gels (NOVEX, San Diego), electrophoretically transferred to a membrane (Immobilon-P; Millipore Corp., Bedford, MA), and immunoblotted as described previously (34). All gels were run under reducing conditions. Densitometric analysis was performed using Kodak 1D software (Eastman Kodak Co.).

Real Time Quantitative Reverse Transcription PCR—Total RNA from wild type and αA-R49C knock-in mouse lenses cortical fractions was isolated according to the manufacturer’s protocol (Qiagen) and treated with DNase I. cDNA was prepared using a kit from Invitrogen. Quantitative reverse transcription-PCR assays of RNA isolated from lenses were performed in 50-μl reactions containing 1× SYBR Green Supermix (Bio-Rad) and 200 μM gene-specific primers for mouse γB, γC, and γD-crystallin genes shown in supplemental Table S1. Assays were performed in triplicate using an I-Cycler (Bio-Rad), and three independent experiments were performed. Primers were designed and synthesized by Integrated DNA Technologies. To optimize the primers, reverse transcription-PCR was performed, and products were run on 1.5% agarose gels to ascertain that a single band of the correct size was obtained. For comparison between wild type and knock-in samples, a standard curve of cycle thresholds for several serial dilutions of RNA
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**RESULTS**

**Generation of \(\alpha\)-A-R49C Knock-in Mice**—A cloning vector containing a point mutation in \(\alpha\)-A-crystallin exon 1 was made (Fig. 1A) and electroporated into 129Sv ES cells, and neomycin-resistant clones were isolated. The targeting vector contained an XhoI restriction site. The mutation in exon 1 of the cyaA gene changed the codon for arginine 49 (CGC) to cysteine (TGC). The DNA from ES clones was sequenced, and the ES clone with no insertion. The upper band (14.5 kb) represents the correctly targeted ES clones. Native \(\alpha\)-A-crystallin gene (12.5 kb) was present in each positive clone, and the appropriate knock-in was the ES clones with the insertion. D, PCR screening of genomic tail DNA confirmed recombination in mice. At the 5’ end a sense flanking primer (pcr1) was paired with an antisense \(\alpha\)-A-crystallin gene intronic primer (pcr2). Primers amplified a 500-bp band from wild type \(\alpha\)-A-crystallin chromosomes, whereas they amplified a 550-bp band from neomycin-deleted knock-in chromosomes. Heterozygous mice amplified both the 500- and 550-bp bands. Absence of the 500-bp band and detection of only the 550-bp band showed homozygosity for the \(\alpha\)-A-R49C mutation.

**TUNEL Staining**—TUNEL labeling was used to examine cell death in lens sections and lens epithelial whole mounts from wild type and \(\alpha\)-A-R49C mouse lenses. Paraffin sections were fixed in 4% paraformaldehyde, pH 7.4, for 30 min and permeabi-

sample was established and then used to calculate the relative abundance levels of mRNA. The expression level of each \(\gamma\)-crystallin mRNA was determined relative to glyceraldehyde-3-phosphate dehydrogenase of the same sample (35, 36).

**TUNEL Staining**—TUNEL labeling was used to examine cell death in lens sections and lens epithelial whole mounts from wild type and \(\alpha\)-A-R49C mouse lenses. Paraffin sections were fixed in 4% paraformaldehyde, pH 7.4, for 30 min and permeabili-

lized for 1 min in 0.1% Triton X-100/ PBS at room temperature, and apoptotic nuclei were detected using a horseradish peroxidase-conjugated TUNEL labeling mix according to the manufacturer’s instructions (Roche Applied Science). TUNEL staining was visualized in an Olympus microscope. The number of TUNEL-positive cells was counted in lens epithelial and fiber cells for each of four sections per lens. Statistical analysis was performed using the Student’s \(t\) test. Lens epithelial whole mounts were stained with fluorescein-conjugated TUNEL reagents as described previously, and TUNEL stained nuclei visualized in a confocal microscope (16, 17).
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A

MS/MS of LLPFLSSTISPYR

Relative Abundance

ST/SP - H2O

Relative Abundance

b4

y12P+

y10

b9-H2O

y8

y7

y5

y4

y3

y2

y1

b10

y10

b12

y12

b13

B

MS/MS of LLPFLSSTISPYYCQLFR

Relative Abundance

b4

b9-H2O

y7

y6

y5

y4

y3

y2

y1

b10

y10

y11

y13

b17

C

Wild type mouse αA-crystallin

1 MDVTIQHPFKRALGFPYPSRLFDQFFEGELFEGYDLPFLSTISPYYRQSFLRTVLDGS
61 ISEVRSDRKFVFLDVKHFSPEDLTVKLEDVEIHGKHERQDDHGYSREFHRYRYP
121 SNVDQSALSCSLSADGMLFTSGPKVQGSDLGHGIRARIPVSREEKPKSSAPSS

R49C mouse αA-crystallin

1 MDVTIQHPFKRALGFPYPSRLFDQFFEGELFEGYDLPFLSTISPYYCQLFRVTVDGS
61 ISEVRSDRKFVFLDVKHFSPEDLTVKLEDVEIHGKHERQDDHGYSREFHRYRYP
121 SNVDQSALSCSLSADGMLFTSGPKVQGSDLGHGIRARIPVSREEKPKSSAPSS
allele. PCR screening was used to genotype the mice. The neomycin cassette was deleted by breeding αA-R49C homozygous knock-in mice with Cre-Ella expressing transgenic mice. PCR genotyping was used to identify wild type, homozygous, and heterozygous mice (Fig. 1D).

Next it was of interest to establish that the mutant lenses produced the R49C mutant αA-crystallin by LC-MS/MS analysis. Fig. 2 shows the MS and fragmentation spectra of the predicted peptide from a combined endoprotease digest (LysC + GluC + trypsin) of the wild type αA-crystallin. The sequence of the doubly charged precursor ion (inset) at m/z = 828.9537 is confirmed by the series of y and b fragment ions (Fig. 2A). The expected endoprotease-digested peptide with m/z of 828.9537 was detected in both wild type and heterozygous knock-in lenses. The expected peptide from the αA-R49C knock-in lens was observed as a doubly charged ion at m/z = 1146.591. The sequence was deduced from the series of y and b fragment ions (Fig. 2B). The signal from the mutant peptide (m/z = 1146.591) was only observed in the mutant lenses (± 2 ppm). The mutant protein loses a trypsin cleavage site as a result of the arginine 49 mutation that lies outside the conserved amino acid sequences of wild type αA-crystallin by LC-MS/MS analysis (Fig. 2C). Instead of the wild type peptide sequence LLPFLSSTISPYR, the sequence LLPFLSTISPYCQSLFR was created. We identified both mutant and wild type peptides by their accurate masses as well as unique sequence (Fig. 2), thus proving that the αA-R49C mutant protein was indeed being expressed in the knock-in mutant lenses in vivo.

Phenotypic and Molecular Changes in αA-R49C Knock-in Mice—The αA-R49C knock-in mice were viable and fertile. Mice were backcrossed to C57BL6 background by breeding. At all ages, cataract severity was the least in wild type. Homozygotes showed a rapid onset that stabilized between 8 and 12 weeks (Fig. 3). Lens opacities were less severe in heterozygous lenses, and a clear dose-response was observed. Stage 3 and 4 lens opacities were observed in 5- and 16-week-old αA-R49C homozygous mice, respectively (Table 1). Interestingly, in the lenses with stage 3 opacities, the lens nucleus was opaque, but the outer cortical fibers were relatively clear. An unexpected phenotype in mouse eyes homozygous for the αA-R49C mutation was severe micro-ophthalmia. αA-R49C homozygous eyes weighed 60–70% less than wild type and heterozygous littersmates, which was mainly due to the decrease in lens weight (Fig. 4).

Mechanism of αA-R49C Effects—Measurement of BrdUrd labeling index in vivo showed a comparable level of cell proliferation in wild type and αA-R49C heterozygous and homozygous lenses (Fig. 5). However, cell death measured by TUNEL staining in the homozygous lens epithelium (Fig. 5 and 6) and lens fiber cells (Fig. 5, E and F) increased suggesting that the cell death is due to apoptosis in the αA-R49C homozygous lenses. TUNEL-positive cells increased 6-fold in the fiber cells (wild type versus αA-R49C homozygous lenses, n = 6 p < 0.01). These data suggest that the smaller αA-R49C homozygous lens is due to a decrease in the number of lens epithelial and fiber cells. To investigate the mechanism by which cell death was induced in the lens epithelial and fiber cell populations of the homozygous αA-R49C mice, we examined whether the presence of the αA-R49C induced changes in expression or activation of molecules involved in cell death and cell survival (Fig. 7). Overall, the signaling changes in the fiber zone were much more pronounced than in lens epithelial cells. In the lens epithelial cells, there was no significant change in the activation of ERK or the expression of Bcl-2, both signals associated with cell survival. Increased activation of Akt and phosphorylation of its target on Bad Ser-136 also occurred, consistent with activation of cell survival signals. The main cell death-associated signal detected in the epithelium of αA-R49C lenses was the inhibition of phosphorylation of Bad at serine 112. Substantial changes in activation or expression of survival and death-inducing signaling molecules in the lens fiber cells of the R49C mice were observed (Fig. 7). The phosphorylation of Bad was suppressed at both serine 112 and 136, a potent signal for apoptosis. Quantitative analysis showed that phosphorylation of Bad at serine 112 decreased 3-fold in the lens epithelium of αA-R49C homozygous lenses. Similarly, the phosphorylation of Bad at serine 112 decreased 3.7- and 5.3-fold in the lens fiber cells of αA-R49C heterozygous and homozygous lenses, respectively, and phosphorylation of Bad at serine 136 decreased 2.4-fold in the lens fiber cells of αA-R49C homozygous lenses. In addition, there was increased expression and phosphorylation of the stress signaling molecule p38. Interestingly, concurrent with activation of pathways associated with cell death, signaling pathways associated with cell survival, including activation of ERK and Akt and induction of Bcl-2, also were induced in the lens fiber cells. However, activation of these pathways appeared ineffective in preventing induction of apoptosis in the lens fiber zone of the αA-R49C mutants. These results suggest that the αA-R49C mutation in the protein chaperone αA-crystallin alters the expression of key cell survival and apoptosis-related proteins, suppressing some and activating others, ultimately resulting in cell death in the homozygous lenses.

Next it was of interest to determine whether the αA-R49C mutant lenses still maintained the molecular markers associ-
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FIGURE 3. Eye and lens phenotypes of αA-R49C knock-in mice. Eyes were dilated and examined by slit lamp in nonanesthetized mice. The dotted line shows the outline of the eye. A–C, 5-week-old mice. A, wild type mice at age 5 weeks had clear lenses. B, heterozygous αA-R49C knock-in mice at 5 weeks displayed low level opacities in the nuclear and posterior regions of the lens. C, homozygous αA-R49C knock-in mice at 5 weeks displayed a severe opacity in the lens nucleus (stage 3) and the small eye phenotype. Note that the opacity lies in the center of the lens, whereas the cortical fibers are relatively clear. The opacities were digitally quantified using the ImageJ program. The light intensity at the cornea was normalized to 100. The plot profiles shown below the slit lamp images demonstrate a gradual increase in light scattering from the wild type to αA-R49C heterozygous to αA-R49C homozygous mice. D–F, 16-week-old mice. D, wild type lens at 16 weeks. E, 16-week-old heterozygous mouse lens had stage 2 lens opacity. F, 16-week-old αA-R49C homozygous mouse displayed micro-opthalmia with severe (stage 4) lens opacities. Note the dotted line showing that the αA-R49C homozygous knock-in eye is significantly smaller than the wild type and heterozygous littermates (D and E). The opacities were digitally quantified using the ImageJ program. The light intensity at the cornea was normalized to 100. The plot profiles shown below the slit lamp images demonstrate a gradual increase in light scattering from the wild type to αA-R49C heterozygous to αA-R49C homozygous mice.

ated with the lens phenotype. We investigated the expression of lens cytoplasmic proteins α-, β-, and γ-crystallins. Increased amounts of αA-crystallin and αB-crystallin were detected in the lens-insoluble fractions, with a greater increase in homozygous than heterozygous lenses (Fig. 8). β-Crystallin expression remained unchanged in the αA-R49C knock-in lenses. In contrast, γ-crystallin was up-regulated in αA-R49C heterozygous and homozygous lenses. Real time quantitative reverse transcription-PCR with gene-specific primers for γB, γC, and γD-crystallin showed that the up-regulation of γ-crystallin occurred at the transcriptional level in heterozygous lenses (Table 2). Furthermore, MIP, a membrane protein important for lens function that is normally found exclusively in lens fiber cell membranes, was detected at the same level in the lens-insoluble fractions (Fig. 8). Immunofluorescence analysis with an MIP antibody showed a severe disturbance of fiber cell morphology in αA-R49C homozygous lenses (Fig. 9), suggesting that αA-R49C expression affects lens fiber cell membrane organization. Interestingly, in the equatorial region it appears that the fiber cells elongate, but later the morphology of the fiber cells becomes altered, and the fiber cell structure is disrupted.

Wild type αA-crystallin has only one cysteine residue in its amino acid sequence, whereas the αA-R49C has two. The second cysteine residue in the N-terminal region could contribute to protein insolubility and cataract by its potential to cross-link with itself and other proteins. Disulfide cross-linking was assessed by extracting lens proteins under nonreducing conditions. Immunoblot analysis with antibodies to αA-crystallin and γ-crystallin detected 60–80-kDa cross-linked immunoreactive bands in the αA-R49C homozygous lenses under nonreducing conditions, but not in the wild type or heterozygous lenses (supplemental Fig. S2).

Because mutations in αA-crystallin are known to affect its chaperone activity and interaction with substrate proteins, co-immunoprecipitation of αA-crystallin and other crystallins was performed with an αA-crystallin monoclonal antibody and immunoblotting with antibodies to αB-crystallin, β-crystallin, and γ-crystallin. The association of αB-crystallin, β-crystallin, γ-crystallin, and β-tubulin increased 2–3-fold in the αA-R49C heterozygous lenses (for example, a 2.3-fold increase for β-crystallin, wild type versus αA-R49C heterozygous, n = 3, p = 0.02), suggesting that the concentration of unstable proteins increases with the mutation (Fig. 10). In contrast, the
interaction of these substrate proteins in αA-R49C homozgyous lenses decreased or remained unaffected. Taken together, these results indicate that αA-R49C causes multiple effects in vivo by changes in interaction with other crystallins, activating lens fiber cell Bcl-2 expression, dephosphorylation of key cell survival protein phospho-Bad, and suppressing or activating other proteins in our heterozygous mutant lenses may be the result of a gain-of-function that increases the interaction of the mutant αA-crystallin with some proteins. This gain-of-function mechanism is supported by the early onset of the cataract in the heterozygous knock-in lenses, because it would take some time for proteins to partially denature in these lenses.

DISCUSSION

We report severe cataracts and small eye phenotype in αA-R49C homozygous lenses and protein insolubility in αA-R49C heterozygous lenses establishing the first knock-in mouse model for hereditary human cataracts caused by a crystallin mutation in vivo. The knock-in mice contained the exon 1 mutation of arginine 49 to alanine mutation decreases the chaperone activity of αA-crystallin (18). Although the loss of chaperone function of αA-crystallin may be one factor that contributes to underlying cataract development in the αA-R49C mutant lenses, the observed high interactions of αA-crystallin with other proteins in our heterozygous mutant lenses may be the result of a gain-of-function that increases the interaction of the mutant αA-crystallin with some proteins. This gain-of-function mechanism is supported by the early onset of the cataract in the heterozygous knock-in lenses, because it would take some time for proteins to partially denature in these lenses.

Analysis of interaction of αA-crystallin with crystallins and β-tubulin in αA-R49C heterozygous lenses by co-immunoprecipitation demonstrated that αA-crystallin senses the presence of unstable β-crystallin, γ-crystallin, and β-tubulin by binding to these substrate proteins at a higher level. Previous studies have shown that αA-crystallin is essential for maintaining unpolymerized tubulin in an assembly competent conformation (43). Studies in the literature show that αA-crystallin forms co-aggregates with γ-crystallin (44, 45). Our results are consistent with these studies and indicate an increase in the interaction between γ-crystallin and αA-R49C in the heterozygous

type lenses showed either mild or no significant abnormality at each age by slit lamp analysis.

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The αA-R49C homozygous mice developed cataracts at birth. The arginine residue at amino acid 49 in αA-crystallin amino acid sequence has been conserved in 56 different species during evolution (19). Investigators have demonstrated that in vitro chaperone activity of αA-crystallin is abolished by replacement of arginine 49 with alanine (18). The αA-R49C knock-in lenses in our study demonstrated a decrease of αA-crystallin and β-crystallin in the lens water-soluble fraction with a concomitant increase of these proteins in the water-insoluble fraction. Mutation of other conserved arginine residues in αA-crystallin has also been shown to reduce protein solubility in vitro, and structural changes in αA-crystallin have been reported with the R116C mutation (37–39). Replacement of the positive charge on arginine 120 in αB-crystallin, (R120G-αB) causes partial protein unfolding, reducing its stability and promoting its aggregation (40). Unlike previous studies however, this work shows the effect of the mutation of an arginine residue in the N-terminal region of αA-crystallin on the solubility of a protein, and it further shows that as αA-crystallin becomes insolubilized, so does its aggregation partner in the lens, αB-crystallin. Several mechanisms may account for this effect. First, the change in isoelectric point and addition of a second cysteine residue in αA-R49C may disrupt the normal conformation of the chaperone, increase its potential for disulfide cross-linking, and increase the co-precipitation of substrate proteins leading to insolubilization (8, 41). An increase in inappropriate disulfide bond formation has also been reported for an arginine to cysteine mutation in γD-crystallin (42). Second, the αA-R49C mutation may increase the dissociation of αA-crystallin oligomer into smaller multimers thereby gaining the capacity to bind substrate proteins. Our results demonstrate an increased interaction of αB-crystallin, β-crystallins, γ-crystallins, and β-tubulin in αA-R49C heterozygous knock-in lenses. Third, the chaperone activity of αA-R49C may decrease and increase the concentration of destabilized substrate proteins. Studies in the literature suggest that the arginine 49 to alanine mutation decreases the in vitro chaperone activity of αA-crystallin (18).
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FIGURE 5. Cell proliferation and death in aA-R49C knock-in eyes. 5-Day-old mice were injected with BrdUrd, and the labeling index was determined. A–C, cell proliferation as measured by in vivo BrdUrd labeling index shows that cell proliferation is qualitatively unchanged in wild type and knock-in lenses. A, wild type lens; B, aA-R49C heterozygous knock-in lens; C, aA-R49C homozygous knock-in lens. The labeling index was determined by dividing the number of BrdUrd-positive cells with the total number of cells in each section. A total of three sections per lens and six lenses per genotype were analyzed. Note that the BrdUrd labeling index was 0.06 ± 0.01 in the wild type, 0.05 ± 0.03 in aA-R49C heterozygous knock-in lenses, and 0.06 ± 0.02 in aA-R49C homozygous knock-in lenses. D–F, TUNEL staining in aA-R49C knock-in lens epithelial and fiber cells. D, wild type mouse lens; E, aA-R49C homozygous knock-in mouse lens; F, quantitative analysis of TUNEL staining in wild type, aA-R49C heterozygous, and aA-R49C homozygous lenses.

FIGURE 6. Cell death in aA-R49C lens epithelial whole mounts. Wild type or aA-R49C homozygous lenses were dissected, and whole mounts of lens epithelial cells were prepared and analyzed by propidium iodide labeling (red) and fluorescein conjugated TUNEL reagent (green). A, wild type lens epithelial whole mount stained with propidium iodide and viewed in the rhodamine channel of the confocal microscope. B, sample in A viewed in the fluorescein channel of the confocal microscope. Note that the wild type sample stained with TUNEL shows no TUNEL-labeled nuclei. C, merged image of the wild type specimen shown in A and B. D, aA-R49C homozygous lens epithelial whole mount stained with propidium iodide shows a cluster of condensed nuclei surrounded by normal appearing nuclei. The sample was viewed in the rhodamine channel of the confocal microscope. E, aA-R49C homozygous specimen in D stained with TUNEL reagent and examined in the fluorescein channel of the confocal microscope. Note that the cluster of condensed nuclei in D was strongly labeled with TUNEL reagent. F, merged image of the aA-R49C homozygous sample shown in D and E.

lenses. Investigators have documented an interaction between the N-terminal domain of βB-crystallin and αA-crystallin (46). This study demonstrated an increase in the interaction between αA-crystallin and βB-crystallin in heterozygous αA-R49C lenses. This observation is somewhat unexpected considering that βB-crystallin is a small heat shock protein and another chaperone, and it is meant to survive stress and protect other proteins. Nonetheless, the increased interaction between βB-crystallin and mutant αA-crystallin has also been reported by other investigators using different methodologies (47).

It is well established that the expression of β-crystallin and γ-crystallin increases dramatically during lens fiber cell differentiation (48). Although no change in β-crystallin expression was observed in the αA-R49C lenses, γ-crystallin was up-regulated as compared with wild type lenses. The mechanism of γ-crystallin up-regulation appears to be an increase in gene transcription in the case of heterozygous lenses, suggesting that expression of the αA-R49C mutant protein may reduce the rate of mRNA turnover (49). Our results suggest that further analysis of mechanisms regulating γ-crystallin expression in the αA-R49C knock-in lenses may be warranted.

The small eye phenotype that we observed in our knock-in mice was due to increased cell death and not reduced proliferation. These results are consistent with our previous findings that gene disruption of αA-crystallin increases lens epithelial cell apoptosis, and that αA-crystallin arginine mutant expression is toxic to lens cells and enhances apoptosis (3, 17, 21). The small eye phenotype was also reported in hereditary human cataract caused by the R116C mutation in αA-crystallin, but its mechanism has not been investigated (2). Although both the autosomal dominant mutations, αA-R49C and αA-R116C, have been shown to enhance apoptosis in lens epithelial cultures, αA-R49C has been found to be more toxic (3, 17). Thus, it is plausible that the small eye phenotype in αA-R49C homozygous mice is due to cytotoxicity of the mutation and the loss of normal anti-apoptotic function of αA-crystallin. αA-crystallin has been shown to act as an anti-apoptotic protein by preventing the activation of caspases (50). Investigators have shown that the lens is important for the proper growth of the anterior segment and the eye (51). This work demonstrates that a decrease in lens weight led to a corresponding decrease in eye weight of the αA-R49C homozygous mice. Furthermore, because the αA-R49C heterozygous mice did not demonstrate the small eye phenotype, our results suggest that the absence of one wild type αA-crystallin allele in the mouse is not sufficient to cause enhanced cell death in vivo. These results further indicate that expression of βB-crystallin and one normal copy of αA-crystallin are sufficient to inhibit apoptosis in vivo but insufficient to prevent protein insolubilization and lens opacity. These results are also consistent with the absence of the small
lens phenotype in the heterozygous αA-crystallin gene knockout mice (52).

Small heat shock proteins are physiological regulators that have co-evolved as integral components of signal transduction pathways. As demonstrated in Figure 7, the expression of cell survival and signaling proteins was altered in αA-R49C knock-in lens epithelial and cortical fiber cells. The lens epithelial and fiber cell fractions were dissected from 16 to 20 WT, αA-R49C heterozygous, and αA-R49C homozygous lenses. Cell lysates were prepared and analyzed by SDS-PAGE and immunoblotting with specific antibodies to Bcl-2, p-Bad Ser-112, p-Bad Ser-136, p-Akt (308), p-p38, total p38, p-ERK, and ERK1/2. Note that wild type lens epithelial cells expressed high levels of Bcl-2, which was maintained in the αA-R49C heterozygous and homozygous lenses. Wild type lens fiber cells expressed low levels of Bcl-2, which increased >4-fold in αA-R49C homozygous lenses but not in αA-R49C heterozygous lenses. Note also that the high expression of p-Bad Ser-112 and p-Bad Ser-136 in lens fiber cells decreased gradually with decrease of WT αA-crystallin and decreased 5.3- and 2.4-fold, respectively, in the αA-R49C homozygous lenses. Expression of p-Akt (308) and p-Akt (473) in lens epithelial cells increased with the αA-R49C mutation. Although p-Akt (308) and p-Akt (473) expression was hardly detectable in WT lens fiber cells, p-Akt (308) and p-Akt (473) increased 3- and 12-fold, respectively, in the αA-R49C lens fiber cells, whereas total Akt increased only 1.2-fold. WT lens epithelial cells expressed high levels of p38 and p-p38, which were slightly reduced (<2-fold) in the αA-R49C homozygous lenses. However, the expression of p38 and p-p38 increased 2–4-fold in the lens fiber cells of αA-R49C homozygous lenses. Note that the antibody to p-p38 identifies the lower p38 band (arrow). Immunoblotting for p-ERK and ERK demonstrated sustained expression and activation of p-ERK and ERK1/2 in lens epithelial cells of WT, αA-R49C heterozygous, and αA-R49C homozygous lenses. Note that WT lens fiber cells expressed one-tenth the level of p-ERK and ERK1/2 of lens epithelial cells. Note also that p-ERK expression was enhanced 3-fold in lens fiber cells of the αA-R49C homozygous lenses.

FIGURE 7. Change in expression of cell survival and signaling proteins in αA-R49C knock-in lens epithelial and cortical fiber cells. Lens epithelial and fiber cell fractions were dissected from 16 to 20 WT, αA-R49C heterozygous, and αA-R49C homozygous lenses. Cell lysates were prepared and analyzed by SDS-PAGE and immunoblotting with specific antibodies to Bcl-2, p-Bad Ser-112, p-Bad Ser-136, p-Akt (308), p-Akt (473), total Akt, p-p38, total p38, p-ERK, and ERK1/2. Note that wild type lens epithelial cells expressed high levels of Bcl-2, which was maintained in the αA-R49C heterozygous and homozygous lenses. Wild type lens fiber cells expressed low levels of Bcl-2, which increased >4-fold in αA-R49C homozygous lenses but not in αA-R49C heterozygous lenses. Note also that the high expression of p-Bad Ser-112 and p-Bad Ser-136 in lens fiber cells decreased gradually with decrease of WT αA-crystallin and decreased 5.3- and 2.4-fold, respectively, in the αA-R49C homozygous lenses. Expression of p-Akt (308) and p-Akt (473) in lens epithelial cells increased with the αA-R49C mutation. Although p-Akt (308) and p-Akt (473) expression was hardly detectable in WT lens fiber cells, p-Akt (308) and p-Akt (473) increased 3- and 12-fold, respectively, in the αA-R49C lens fiber cells, whereas total Akt increased only 1.2-fold. WT lens epithelial cells expressed high levels of p38 and p-p38, which were slightly reduced (<2-fold) in the αA-R49C homozygous lenses. However, the expression of p38 and p-p38 increased 2–4-fold in the lens fiber cells of αA-R49C homozygous lenses. Note that the antibody to p-p38 identifies the lower p38 band (arrow). Immunoblotting for p-ERK and ERK demonstrated sustained expression and activation of p-ERK and ERK1/2 in lens epithelial cells of WT, αA-R49C heterozygous, and αA-R49C homozygous lenses. Note that WT lens fiber cells expressed one-tenth the level of p-ERK and ERK1/2 of lens epithelial cells. Note also that p-ERK expression was enhanced 3-fold in lens fiber cells of the αA-R49C homozygous lenses.

FIGURE 8. Expression of α, β, and γ-crystallins and MIP in wild type and αA-R49C knock-in lenses. 8-Week-old lenses were divided into soluble and insoluble fractions and analyzed by immunoblot analysis with antibodies to αA-crystallin, αB-crystallin, total β-crystallin, total γ-crystallin, and MIP. The expression of αA-crystallin and αB-crystallin increased significantly in the insoluble fractions of the R49C knock-in lenses. γ-Crystallin but not αB-crystallin or β-crystallin expression was up-regulated in soluble fractions of αA-R49C heterozygous and homozygous lenses. MIP was detected as a 27-kDa band. The expression of MIP was the same in wild type, αA-R49C heterozygous, and homozygous lenses when corrected for protein loading in the αA-R49C homozygous lane.
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our studies have demonstrated an association of αA-crystallin with α6 integrin in membranes of lens fiber cells, consistent with the linkage of this chaperone with integrin survival signaling (55). Changes in abundance and relative levels of chaperones can change intracellular signaling pathways with some pathways becoming favored and others being suppressed or constitutively activated. Examination of pathways with some pathways becoming favored and others

ative levels of chaperones can change intracellular signaling integrin survival signaling (55). Changes in abundance and rel-

tion of fibers (53, 54). Our studies have demonstrated an associa-

tion of α-crystallin with α-crystallin (56, 57). In order for

Bcl-2 to have pro-survival functions, it must be released from

the protein Bad, a central protein which in its phosphorylated form is necessary for survival of many cells (58). In the lens fiber cells of αA-R49C knock-in heterozygous and homozygous lenses, both p-Bad Ser-112 and p-Bad Ser-136 were strongly

up-regulated in the cortical fiber cells of these lenses. Bcl-2 has been shown to be up-regulated in lens epithelial cells exposed to oxidative stress, and members of the Bcl-2 family have been shown to be sequestered by αA-crystallin (56, 57). In order for

Bcl-2 to have pro-survival functions, it must be released from

the protein Bad, a central protein which in its phosphorylated form is necessary for survival of many cells (58). In the lens fiber cells of αA-R49C knock-in heterozygous and homozygous lenses, both p-Bad Ser-112 and p-Bad Ser-136 were strongly

down-regulated. These findings indicate that the mechanism of cell death that we are seeing in the αA-R49C mutant lenses is likely to be the result of dephosphorylation of Bad. Because Bad

is phosphorylated by Akt and ERK, the fact that both Akt and ERK were enhanced in αA-R49C lens fiber cells suggests that p-Bad is also being targeted by other kinases. Other kinases that have been implicated in survival signaling have been proposed to mediate Bad phosphorylation, including Rsk, PAK, p70 S6K, and cAMP-dependent protein kinase (59, 60). Moreover, p38 and phospho-p38, known to be important for cell survival or apoptosis depending on the cell type, were enhanced in the αA-R49C knock-in lens fiber cells (61, 62). Activation of p38 has been shown to lead to induction of cataract (61).

In contrast to its low expression in the wild type lens fiber cells, the pro-survival protein Bcl-2 was expressed at high levels in lens epithelial cells, and its levels were maintained in the

αA-R49C heterozygous as well as homozygous knock-in lenses; however, phosphorylated Bad was expressed at very low levels in lens epithelial cells of each of the genotypes. These data show that although lens cells in the αA-R49C lenses express elevated levels of some survival factors, the cells still die. The results are consistent with our studies demonstrating that numerous cell survival pathways are induced in lens cells undergoing apoptosis (35). Studies in the literature demonstrate that cells continu-

ually integrate signals from apoptosis-inducing and apoptosis-suppressing pathways as a means to coordinate their response to stressful conditions (35, 63, 64). Taken together, our in vivo cell proliferation, TUNEL, and cell signaling and survival stud-

ies show that the loss of αA-crystallin function, due to expression of αA-R49C mutant protein in vivo, is cytotoxic to the lens epithelial and fiber cells.

Investigators have shown that in addition to its more typical cytoplasmic distribution, αA-crystallin is also associated with

TABLE 2

Quantitative reverse transcriptase-PCR analysis of γ-crystallin transcripts (Crygb, Crygc, and Crygd) in mouse lenses

| Relative intensity (Cryg/GAPDH)* | Wild type | αA-R49C | Fold increase (αA-R49C/wild type) |
|----------------------------------|-----------|---------|----------------------------------|
| Crygb                            | 2.19 ± 0.30 | 5.35 ± 0.42 | 2.4 |
| Crygc                            | 3.65 ± 0.27 | 10.23 ± 0.64 | 2.8 |
| Crygd                            | 0.56 ± 0.28 | 4.55 ± 0.34 | 8.1 |

* GAPDH is glyceraldehyde-3-phosphate dehydrogenase.

FIGURE 9. Immunofluorescence analysis of MIP expression in wild type and αA-R49C homozygous knock-in lenses. Lens slices were cut in the equatorial plane and stained with an antibody to lens MIP (AQP0) to visualize fiber cell membranes. Sections of wild type (A, C, E, and G) and homozygous (B, D, F, and H) lenses are shown. Low magnification images of wild type (A) and αA-R49C homozygous lenses (B) show the dramatic decrease in lens size in the mutant. Note that the αA-R49C homozygous lenses were significantly more fragile and susceptible to tearing during processing. C and D, visualization of fiber cell membranes in the onset of differentiation (cell elongation) region of wild type (C) and αA-R49C homozygous (D) lenses. Nuclei are indicated by bold arrows. The organization of the fiber cells in these equatorial sections is different, with the neat packing of membranes of the wild type lenses (small arrowheads). E and F, in contrast to the neat parallel organization of lens fiber cells in the wild type lens (E), fiber cells of the αA-R49C homozygous lenses (F) were highly disorganized. The distance from the center of the lens was 400 μm. G and H, posterior region of the lens. Lens fiber cells demonstrate a neat hexagonally packed arrangement in this section of the wild type lens (G), in contrast to the highly disorganized pattern in the αA-R49C homozygous lens (H).
are known to stabilize the packing arrangement of lens fibers (67–70). Changes in adhesion complexes occur at the stage between completion of fiber cell elongation and degradation of membrane-bound organelles (71). The dramatic fragility of the αA-R49C homozygous lenses suggests a destabilization of these adhesions. Although lens fiber elongation per se appears to occur in the αA-R49C knock-in homozygous lenses, this study demonstrated dramatic structural alterations in fiber cell morphology and is consistent with the reported interactions between membranes and αA-crystallin in lens fiber cells (72, 73).

In summary, this study clearly establishes the first mouse knock-in model for hereditary cataract caused by a point mutation in αA-crystallin. αA-R49C heterozygosity demonstrated protein insolubility and lens opacities that were apparent at an early postnatal age, whereas homozygosity exhibited a small eye and lens phenotype in addition to severe cataracts. These data show that the presence of mutant αA-R49C is detrimental to the development and emergence of the transparent phenotype, and convincingly show that αA-crystallin has non-refractive functions modulated by the genetics of lens epithelial and fiber cells (74). The dramatic effects that we are seeing in cell structure as a result of αA-R49C mutation are the subject of future studies. Using our knock-in approach, it would be interesting to determine whether other αA-crystallin mutations will have the same or other effects on the eye.

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