αB-Crystallin in Lens Development and Muscle Integrity: A Gene Knockout Approach

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PURPOSE. To study the role of αB-crystallin (αB) in the developing lens and its importance in lens structure and function.

METHODS. Gene targeting in embryonic stem cells was used to generate mouse lines in which the αB gene and its protein product were absent. Gene structure and expression were characterized by genomic Southern blot, immunoblot, and Northern blot analyses, and two-dimensional gel electrophoresis. The gene knockout mice were screened for cataract at cataract with slit lamp biomicroscopy, and dissected lenses were examined with dark-field microscopy. Lenses and other tissues were analyzed by standard histology and immunohistochemistry. Chaperone activity was determined by heating lens homogenate supernatants and measuring absorbance changes.

RESULTS. In an unexpected result, lenses in the αB gene knockout mice developed normally and were remarkably similar to wild-type mouse lenses. All the other crystallins were present. The thermal stability of a lens homogenate supernatant was mildly compromised, and when oxidatively stressed in vivo with hyperbaric oxygen, the knockout lenses reacted similarly to wild type. In targeting the αB gene, the adjacent HSPB2 gene, which is not expressed in the lens, was also disrupted. Loss of αB and/or HSPB2 function leads to degeneration of some skeletal muscles.

CONCLUSIONS. αB is not essential for normal development of a transparent lens in the mouse, and therefore is more dispensable to the lens than the closely related αA-crystallin. It may play a small role in maintaining transparency throughout life. αB and/or the closely related HSPB2 is required to maintain muscle cell integrity in some skeletal muscles.

The α-crystallins are members of the small heat shock family of proteins that, together with the β- and γ-crystallins, comprise the major water-soluble proteins of the vertebrate ocular lens. The two α-crystallin proteins, αA- and αB-crystallin (hereafter known as αA and αB), are approximately 60% identical with one another and share many common properties in vitro, such as autokinase activity,1 interaction with cytoskeletal proteins,2–5 DNA binding,6 and the ability to act as molecular chaperones.7 However, there are differences between the two proteins. Although both α-crystallins show sequence similarity to small heat shock proteins (sHSPs), only αB is inducible by stress.8 Also, αA is expressed at very high levels in the lens and is found in trace amounts in only a few nonlenticular tissues.9–12 whereas αB, although it is most highly expressed in the lens, is found at significant levels in a number of tissues.13–15 αB is particularly abundant in adult heart and skeletal muscle, and its message has been detected very early in the developing mouse heart and somites.16–17

The α-crystallins were originally thought to be lens-specific structural proteins. However, the discoveries that αB is expressed outside the lens and that it is stress inducible have brought broader attention to the α-crystallins and to their potential usefulness for elucidating the functions and evolution of stress proteins. In addition, there is a growing list of human diseases in which αB has been shown to be misexpressed, mislocalized, or otherwise involved.18 One disease, an autosomal dominant desmin-related myopathy, was recently shown to result from a missense mutation in the human αB gene. This disorder is characterized by adult-onset muscular weakness, cardiomyopathy, and cataracts.19,20 These clinical findings and the observation that αB is expressed very early in the developing heart and skeletal muscles suggest a critical physiological role for αB.

To elucidate the in vivo functions of α-crystallins, we generated mice with targeted disruptions of the genes that encode the αA and αB proteins. We previously reported that homozygous αA knockout mice have smaller lenses than those of wild-type littermates and that progressive opacifications develop in the knockout lenses that apparently result, at least in part, from the presence in lens fiber cells of inclusion bodies that contain high concentrations of αB.21 Herein, we present the initial characterization of mice with a targeted deletion that disrupts both the αB gene and the adjacent gene, HSPB2, which is an ancient duplication of αB.22 The existence of the HSPB2 gene, which lies approximately 1 kb upstream of αB, was reported after we produced the αB knockout mice. Similar to αB, HSPB2 is expressed in skeletal and cardiac muscles where it is often localized to the Z lines and has also been shown to associate with and activate myotonic dystrophy protein kinase.23 Unlike αB, HSPB2 is not expressed in the lens.22 Mice without both αB and HSPB2 are surprisingly viable and fertile and have no obvious perinatal defects, and their lenses remain as transparent as those of wild-type mice. However, as they become older, αB/HSPB2 homozygous knockout mice show postural defects and other health problems that appear to stem from progressive myopathy.

MATERIALS AND METHODS

Generation of αB/HSPB2 Gene Knockout Mice

A λ phage clone spanning approximately 16 kb of the αB/HSPB2 locus was isolated from a 129sv mouse genomic library (Stratagene, La Jolla, CA; obtained under the terms of US government contract N01-NS-3-0006). This clone contained both the αB and HSPB2 genes. The fragment was introduced into two embryonic stem cell lines (129SV.SV129 and 129Sv/J) in which the homologous endogenous loci had been replaced by a selectable marker, neomycin phosphotransferase. The clones were then injected into C57BL/6J blastocysts to generate chimeric mice. Male chimeras were then mated to C57BL/6J mice to obtain flp transgenic animals that express the site-specific recombination enzyme flp, which is disrupted in the αB gene at a 

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Supported in part by National Institutes of Health Grant EY02027 (FGJ) and Core Center Grant EY05230.

Submitted for publication April 4, 2001; revised July 19, 2001; accepted August 6, 2001.

Commercial relationships policy: N.

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A targeting vector (Fig. 1A) was constructed by ligating two restriction fragments: a 3-kb BamHI fragment, encompassing a portion of exon 3 and the 3′ flanking sequences of the αB gene and a 5-kb NcoI/NarI fragment encompassing a portion of exon 2 and the 3′ flanking sequences of the HSPB2 gene, into the vector pPNT on opposite sides of the phosphoglycerate kinase-neomycin phosphotransferase (PGK/neo) gene cassette (Fig. 1A). The targeting vector also included the PGK/herpes simplex virus thymidine kinase (PGK/HSVtk) gene cassette for negative selection. Electroporation of J1 embryonic stem cells, selection and screening of targeted cells, and blastocyst microinjection were performed as previously described. Two independent embryonic stem (ES) cell clones were used to generate two lines of knockout mice. DNAs from ES cells and knockout mice were analyzed by PCR, using primers spanning, but not encoded within, the shorter arm of the knockout vector and by Southern blot, using the 3-kb BamHI fragment (Fig. 1A; triple line) as a probe. Once stable lines of knockout mice were established, a simpler PCR protocol was used for genotyping. PCR using three primers, 5′-TAGGTATGCTGAAGTTCCACAGGAAGTACC-3′, 5′-GGAGTTCCACAGGAATGACAGG-3′, and 5′-TGGAAGTTGTCGCTAGG-3′, in a 4:1:1 molar ratio produced a 310-bp product with the wildtype allele and a 600-bp product with the knockout allele. All work with mice conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Western Blot Analysis of Lens and Muscle Proteins**

Soluble and insoluble lens protein samples were prepared as previously described. Mouse skeletal muscles were homogenized in TE buffer (10 mM Tris; 1 mM EDTA) with 2.5% SDS, and protein concentrations were determined using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). Muscle proteins were separated on 12% SDS-polyacrylamide gels and then transferred to nitrocellulose, by a semi-dry transfer apparatus (Semi-Phor; Hoefer Scientific Instruments, San Francisco, CA). The blots were incubated with a 1:5000 dilution of polyclonal antiserum to recombinant human αB-crystallin (a gift from Joseph Horwitz, Jules Stein Eye Institute, University of California at Los Angeles School of Medicine). Protocols for antibody incubation and detection have been described.

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**Figure 1.** Targeted disruption of the αB and HSPB2 genes. (A) Structures and relative orientation of the HSPB2 and αB genes in a wildtype mouse, gene-targeting construct, and structure of the targeted locus. **Double and triple lines** beneath the wildtype locus indicate the 3-kb HpaI/XbaI and the 3-kb BamHI restriction fragments used as hybridization probes for Northern and Southern blot analyses, respectively. (B) Southern blot analysis of genomic DNA from wild-type (+/+), heterozygous (+/−), and homozygous (−/−) mice. Bands of the expected sizes for the targeted allele were seen in the +/− and −/− samples digested with either HpaI (15 kb) or EcoRI (13 kb). (C) Immunoblot analysis of αB-crystallin in lens proteins from +/+ , +/− , and −/− mice showed that αB is absent in −/−, and its levels were decreased in +/− mice. A minor cross-reactivity of this antibody toward a slightly smaller protein was observed in the knockout lens. (D) Northern blot analysis of lens and muscle tissues from wild-type (+/+ ) and homozygous knockout (−/−) mice, by using a probe specific for HSPB2 (double line in A). HSPB2 mRNA was not detected in any tissues from knockout mice, nor in the lens of wild-type mice. The blot was reprobed with a β-actin probe that cross-hybridizes with α-actin in muscle tissues (D, bottom). N, NarI; B, BamHI; E, EcoRI; H, HpaI; H, heart; G, gastrocnemius muscle; L, lens; S, soleus muscle; P, plantaris muscle.
Northern Blot to Detect HSPB2 mRNA

Northern blot analysis was performed using standard protocols.23 RNA was extracted from lens, heart, soleus, plantaris, and gastrocnemius muscles of 16- to 17-week-old knockout and wild-type mice. Total RNA (10 μg for lens, heart and gastrocnemius; 5 μg for soleus and plantaris) was used, and before blots were formed, a gel lane containing RNA size markers (Life Technologies, Rockville, MD) was removed and stained. The blot was probed with a 3.5 kb Hpal/Xbal genomic fragment (Fig. 1A; double line), spanning the entire HSPB2 gene and approximately 0.8 kb of its 3′ flanking sequence, and then reprobed with a β-actin probe (Oncor, Rockville, MD).

Slit Lamp Biomicroscopy

Mouse eyes were dilated with eye drops containing 1% tropicamide followed several minutes later by eye drops containing 2.5% phenylephrine hydrochloride. Approximately 15 minutes later, the mice were anesthetized with an intraperitoneal injection of 0.015 to 0.017 ml 2.5% 2,2,2-tribromoethanol per gram body weight and immediately examined with a slit lamp. The slit illumination was always at a 45° angle to the observation optics.

Histology

Eyes were removed, gently pierced in the limbal region, and fixed in 4% buffered glutaraldehyde for 20 minutes, followed by 4% buffered formaldehyde or 4% buffered formaldehyde alone. For a milder treatment, eyes were occasionally fixed in 4% buffered paraformaldehyde for 25 minutes, rinsed for 30 minutes in PBS, and placed in 80% ethanol for several days. They were embedded in methacrylate or paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Hindlimbs and spine regions were fixed in 4% buffered formaldehyde, decalcified, embedded in methacrylate or paraffin, sectioned, and stained with H&E. Tongue was similarly processed, but the decalcification step was omitted.

Protection from Heat-Induced Denaturation Assay

Pairs of lenses from wild-type, heterozygous and homozygous αB knockout, and homozygous αA knockout mice were disrupted in 1 ml of buffer (0.1 M sodium phosphate [pH 7.4]; 0.1 M sodium chloride). The homogenate was centrifuged for 10 minutes at 4°C at 14,000g in a microfuge. The supernatant was removed and the pellet was resuspended with another 1 ml buffer. After centrifugation, each supernatant was pooled with the first. Protein concentrations were determined by BCA assay (Pierce) and samples were diluted to 3.1 mg/ml

Two-Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis and image analysis were performed, essentially as previously described.29 Briefly, lenses frozen on dry ice immediately after dissection and maintained at −70°C were thawed in a protease inhibitor mix (P8340; Sigma, St. Louis, MO) then homogenized in 9 M urea, 2% NP40, 10 mM diithiothreitol (DTT), 2% ampholytes (Resolyte 3.5-10), and centrifuged at 16,000g for 20 minutes. The supernatant was run in the first-dimension isoelectric focusing strip, using dry-strip gels (pH 3–10 nonlinear; Immobiline DryStrip; Amersham Pharmacia Biotech, Piscataway, NJ), and in the second dimension, using 15% to 18% SDS polyacrylamide gradient gel. Gels were stained with Coomassie blue, cleared with water, scanned on a personal densitometer (Molecular Dynamics, Sunnyvale, CA), and analyzed by computer (Phoretix, ver. 5.0; Phoretix International, Newcastle-upon-Tyne, UK) software.

Immunohistochemistry

Tissues used for immunohistochemistry were fixed in 4% buffered paraformaldehyde. Spine and hindlimb tissues were then decalcified. All tissue preparations were embedded in paraffin and sectioned onto silane-coated slides. Immunohistochemistry was performed using immunoperoxidase reagents in avidin-biotin complex (ABC) and substrate kits (Vectastain Universal Elite and VIP, respectively; Vector Laboratories, Burlingame, CA), according to the manufacturer's instructions. Serial sections were immunostained, and, because mouse monoclonal antibodies were being used with mouse tissues, one slide in each experiment was run without any primary antibody, as a control for nonspecific detection by the anti-mouse IgG secondary antibody. One slide was also stained with H&E. Monoclonal antibodies against myosin heavy chains, (MHC fast and MHC slow; Sigma) were used at 1:500 dilution, and the monoclonal antibody against desmin (Dako, Carpinteria, CA) was used at dilutions of 1:20 to 1:75.

Transmission Electron Microscopy

Eyes were removed, gently pierced in the limbal region, and fixed in freshly prepared 2.5% glutaraldehyde and 2.5% paraformaldehyde in 80 mM cacodylate buffer (pH 7.2). After fixation, the tissues were equilibrated in cacodylate buffer for at least 24 hours and then postfixed with 2% OsO4 and embedded in Epon (Roth, Karlsruhe, Germany). Ultrathin sections were contrasted with lead citrate and uranyl acetate and examined with a transmission electron microscope (EM 902; Carl Zeiss, Oberkochen, Germany).

RESULTS

Generation of αB/HSPB2 Knockout Mice

Figure 1A shows the strategy that was used to knock out the αB gene and concomitantly the adjacent HSPB2 gene. All the protein-coding sequences through the middle of the third and final exon of αB and 1.6 kb of DNA encompassing the putative transcriptional regulatory sequences for αB and HSPB2 as well as most of the HSPB2 protein-coding sequences were eliminated in the targeted chromosome. Genomic Southern blot analysis demonstrated the expected rearrangements of the αB and HSPB2 genes in heterozygous and homozygous knockout mice (Fig. 1B). Deletion of an EcoRI site in the targeted allele resulted in an increase from 9 to 13 kb of the probe-containing EcoRI fragment, and replacement of the shHSP genes with the shorter PGK/neo cassette decreased the size of the probe-containing Hpal fragment from 18 to 15 kb. Western blot analysis of soluble and insoluble proteins from the lens, the tissue with the highest expression level of αB, confirmed that the full-length form and two C-terminally truncated forms of αB were all decreased in heterozygous and absent in homozygous knockout mice (Fig. 1C). Further evidence for the absence of αB is the absence of an αB signal in immunoblots of skeletal muscle and heart (not shown) and the loss of immunologic tolerance to αB as a self-antigen in knockout mice (Igal Gery, personal communication, November 1996).

The closely linked HSPB2 gene was discovered subsequent to the production of these knockout mice, and because available sequence data22 suggested we had disrupted this gene also, Northern blot analysis was performed to confirm this. Figure 1D shows that HSPB2 mRNA, which was previously detected in adult heart and skeletal muscles but not in lenses of wild-type rats,22 was absent from heart and skeletal muscles of homozygous αB/HSPB2 knockout mice, but was present in

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wild-type mouse muscle tissues, confirming that the \textit{HSPB2} gene, in addition to the \textit{\alpha b} gene, was functionally inactivated. Figure 1D also confirms that HSPB2 is not expressed in the lenses of normal mice.

\textit{\alpha b/HSPB2}^{-/-} mice are born at nearly normal Mendelian ratios from matings between heterozygous (+/-) animals (20\% of 200 such offspring were --). At birth, the knockout mice exhibit no obvious mutant phenotypes.

**Characterization of Lens Structure and Development**

Analysis of lenses from 11-week-old mice revealed average lens weights of 5.5 \pm 0.2, 5.3 \pm 0.2, and 5.1 \pm 0.2 mg for wild-type, heterozygous, and homozygous \textit{\alpha b}-crystallin/\textit{HSPB2} knockout mice, respectively. Measurement of the equatorial and axial dimensions of whole eyes and lenses from the same mice revealed no significant differences (not shown). This is in stark contrast to \textit{\alpha A} knockout mice in which the lens weight is reduced by approximately 30\% and the axial and equatorial dimensions are decreased by approximately 15\%.21

The slit lamp micrographs (Figs. 2A, 2B) show little if any difference in light scattering between the wild-type and knockout lenses. There is a basal level of light scattering in the lenses of 129Sv mice that manifested itself in slit lamp examination as an apparent opacity at the edge of the lens opposite the slit illumination. This may have been due to the recently discovered absence of the lens-specific beaded filament cytoskeletal proteins, cp49 and filensin, in lenses of 129Sv strains of mice.27 Age-matched knockout mice, particularly at older ages, appear to have slightly more of this light scattering. Examination of dissected lenses with dark-field illumination (Figs. 2C, 2D) confirms the similarly low light scattering in wild-type and \textit{\alpha b} knockout lenses, which contrasts with the high degree of light scattering in the \textit{\alpha A} knockout lenses. Histologic examination of lenses revealed no obvious differences between wild-type and \textit{\alpha b} knockout specimens (Fig. 3). In contrast, mice in which the closely related \textit{\alpha A} gene is disrupted (Figs. 3E, 3F) show development of lens opacities before 7 weeks of age, and histologic abnormalities even earlier,21 which progress in severity with age (Figs. 2A, 2C, 2D).

Certain abnormalities were observed in both wild-type and \textit{\alpha b} knockout lenses, depending on the method of fixation used. Fixation with 4\% buffered formaldehyde alone (Figs. 3G-J) caused an invagination of the lens at the equator that was similar in wild-type (Fig. 3G) and \textit{\alpha b} knockout (Fig. 3I) mice. When eyes were fixed with 4\% buffered glutaraldehyde for 20 minutes followed by 4\% buffered formaldehyde, small vacuoles were observed in the outer cortex of lenses from both wild-type (Figs. 3K, 3L) and \textit{\alpha b} knockout (Figs. 3M-P) mice. Samples fixed with buffered formaldehyde alone (Figs. 3G-J) or formaldehyde for 25 minutes followed by 80\% ethanol (not shown) did not exhibit these vacuoles. Because these vacuoles appeared with only one method of tissue fixation and independently of the presence or absence of \textit{\alpha b}, they may have been artifactual. Cytoplasmic inclusion bodies present in the lens nuclei of \textit{\alpha A} knockout mice (Fig. 3F) were not observed in the lenses of \textit{\alpha b} knockout mice (Fig. 3D). These results clearly demonstrate that the lens can much more easily accommodate the loss of \textit{\alpha b} function than it can the loss of \textit{\alpha A} function. Because the \textit{HSPB2} gene is not expressed in the lens22 (Fig. 1D), its disruption is not expected to affect the lens.

**Crystallin Expression in Knockout Lenses**

Because the lenses of \textit{\alpha b} knockout mice appear normal and transparent, we suspected that the distribution of other major crystallins was not significantly affected by the absence of \textit{\alpha b}. Two-dimensional gel electrophoresis of lens proteins (Fig. 4) followed by image analysis, which detects spots too faint to be seen by the naked eye, revealed that all the major and minor crystallin spots present in wild-type mice, except, of course, for \textit{\alpha b} and modified forms of \textit{\alpha b}, were also present in the knockout mice. There were no new or additional crystallin spots detected in the knockout mice. \textit{\alpha b}, its mono- and di-phosphorylated forms, and the phosphorylated and unphosphorylated forms of the \textit{\alpha b} C-terminal truncation (amino acids 171 to 175 removed) are all absent in the \textit{\alpha b} knockout lens.
evident throughout the nuclear and inner cortical fiber cells of similar to wild-type lenses, did not have the inclusion bodies that were with vacuoles in the cortex of both wild-type and knockout mouse lenses. (G) Bow region and (H) epithelial region of a 10-month-old wild-type mouse lens that had been fixed in 4% formaldehyde. (I) Bow region and (J) epithelial region of a 10-month-old αB knockout mouse lens that had been fixed in 4% formaldehyde. The wild-type and αB knockout lenses were similar, with an artificial invagination at the equator. (K) Bow region and (L) epithelial region of a 10-month-old wild-type lens that had been fixed in glutaraldehyde followed by formaldehyde. (M, O) Bow regions and (N, P) epithelial regions of 10-month-old αB knockout mouse lenses that had been fixed in glutaraldehyde followed by formaldehyde. The lenses were similar with vacuoles in the cortex of both wild-type and αB knockout. wt, wild-type; ko, knockout. Scale bar, (G–P) 100 μm.

**Protection against Stress in the Lens**

Because αB is an sHSP and molecular chaperone, we investigated whether lenses without αB were more susceptible to damage. The ability of lens proteins to resist heat-induced denaturation was studied (Fig. 5). When the soluble fraction of lens homogenate from wild-type mice was heated to 54°C, there was little increase in turbidity of the solution over the 60-minute experiment, indicating that most proteins remained soluble and that these lenses have thermal protective capacity. The lens homogenate supernatant without αB showed a slow increase in turbidity (and therefore, protein denaturation) beginning at 30 minutes and reached twice the level in wild-type lenses at 60 minutes, suggesting that the overall protective activity in the αB knockout lens was mildly impaired (Fig. 5A). When the lens homogenate supernatants were heated to 65°C, the difference between wild-type and αB lenses became more pronounced, with turbidity of the knockout homogenate supernatant reaching three times that of wild type at 60 minutes (Fig. 5B). There was still a high degree of thermal protection in the αB knockout lenses compared with αA knockout lenses, which exhibited almost no protective capacity, with much of the protein precipitating after 15 minutes (Fig. 5B).

We also tested in vivo, the ability of αB knockout lenses to resist oxidative stress. Exposure of humans and animals to hyperbaric oxygen has been shown to induce an increased level of lens nuclear light scattering or nuclear cataract. Repeated exposure of mice to hyperbaric oxygen (2.5–3.2 atmospheres of O₂ for 2.5–3 hours per exposure, three exposures per week) over a period of 5.5 months induced increased lens nuclear light scattering equally well in wild-type and knockout mice (not shown), suggesting that in vivo, αB does not play a major role in protecting the lens against oxidative damage. This is in agreement with the results of Kannan et al., who demonstrated that the level of reduced glutathione (GSH) in lenses without αB is equal to that in wild-type lenses, whereas lenses without αA exhibit a severe decrease in GSH level, and with the results of Andley et al., who demonstrated that αB is significantly less effective than αA in protecting lens cells against stress. Development of lens opacity in αA knockout mice at an early age, even in the absence of added oxidative stress, precludes similar hyperbaric oxygen experiments from providing useful data on αA knockout mice.

**FIGURE 3.** Histologic examination of lenses from wild-type and knockout mice. H&E-stained sections of lenses from (A) 7.5-week-old wild-type, (C) 9-week-old αB knockout, and (E) 8-week-old αA knockout mice. (B, D, F) Higher magnification of lens regions delimited by rectangles in (A), (C), and (E), respectively. αB knockout lenses, similar to wild-type lenses, did not have the inclusion bodies that were evident throughout the nuclear and inner cortical fiber cells of αA knockout lenses. (G) Bow region and (H) epithelial region of a 10-month-old wild-type mouse lens that had been fixed in 4% formaldehyde. (I) Bow region and (J) epithelial region of a 10-month-old αB knockout mouse lens that had been fixed in 4% formaldehyde. The wild-type and αB knockout lenses were similar, with an artificial invagination at the equator. (K) Bow region and (L) epithelial region of a 10-month-old wild-type lens that had been fixed in glutaraldehyde followed by formaldehyde. (M, O) Bow regions and (N, P) epithelial regions of 10-month-old αB knockout mouse lenses that had been fixed in glutaraldehyde followed by formaldehyde. The lenses were similar with vacuoles in the cortex of both wild-type and αB knockout. wt, wild-type; ko, knockout. Scale bar, (G–P) 100 μm.

**FIGURE 4.** Two-dimensional gel electrophoresis analysis of proteins in αB knockout lenses. Proteins from 10-week-old wild-type (A) and 11-week-old αB knockout (B) mice were analyzed. (A) Wild-type profile shows αB spot (large arrowhead) and several αB phosphorylation products and C-terminal truncations (arrow). (B) The main αB spot and all the minor spots corresponding to modified αB are absent in the αB knockout lenses; vacant positions are indicated by arrowhead and arrow as in (A). All other major and minor spots, including those not visible to the naked eye, but detected by the computer software, are present in both wild-type and αB knockout lenses.
Health Problems in Aging Knockout Mice

The growth curves of male and female knockout mice paralleled those of wild-type 129Sv until approximately 40 weeks of age (data not shown); but thereafter, the knockout mice consistently lost weight and eventually lost most of their body fat. The mice also displayed a hunched posture resulting from development of a severe curvature of the spine (kyphosis) that could be readily observed in radiograms of 12-month-old mice (Fig. 6). Histologic examination of the hunched mice revealed degenerative osteoarthritus of the intervertebral facet joints. Although not uncommon in much older wild-type mice, this process appears to be greatly accelerated in the knockout mice. Because both αB and HSPB2 are expressed in muscle cells, we suspected that the underlying cause of the observed kyphosis was related to altered function of muscles associated with the axial skeleton. In histologic examination of 65-week-old knockout mice exhibiting the hunched posture, the heart appeared normal (not shown), but severe degeneration of some skeletal muscles was readily apparent (Fig. 7) with the most severely affected muscles being in the posterior tongue (Figs. 7B, 7D), head, and surrounding the axial skeleton (Figs. 7F, 7H). Limb muscles were affected to a lesser extent (not shown). None of these degenerative changes was observed in the skeletal muscles of age-matched heterozygous (not shown) or wild-type mice (Figs. 7A, 7C, 7E, 7G).

In an area of the posterior tongue of a 65-week-old knockout mouse, degenerated muscle was nearly completely replaced by fatty tissue (Figs. 7B, 7D). The few muscle cells remaining were quite abnormal and probably would have disappeared soon thereafter. Axial skeletal muscles exhibited a variety of alterations that may represent a progression of the degenerative processes. These include migration of nuclei into the sarcoplasm, hyalin degeneration of the sarcoplasm, vacuolization of the sarcoplasm, infiltration of macrophages, fibrosis, and fatty replacement of muscle cells. Several of these abnormalities can be seen in Figures 7D and 7H. Muscle weakness in the tongue and head regions probably leads to an impaired ability to obtain nourishment, accounting for the loss of weight and body fat observed in older mice; and decreased axial skeletal muscle function probably leads to the accelerated degenerative vertebral osteoarthritis and hunched posture observed in older knockout mice.

Because the muscle degeneration in older mice was severe and apparently the consequence of chronic muscular dystrophy, we examined knockout mice at several younger ages (not shown). At 7 weeks of age muscles appeared relatively normal, exhibiting only very minimal axial muscular dystrophy. By 20 weeks of age, the axial muscular dystrophy had progressed in severity, exhibiting some occasional fibrosis, fatty infiltration, and myositis, suggesting the chronic nature of this condition. By this age, muscles of the head were also affected, and the diaphragm and select hindlimb muscles (e.g., the soles) were mildly affected. At 40 weeks of age, the accumulated damage to the axial muscles was more severe, with fibrosis and fatty infiltrates more pronounced. Muscles of the head, particularly those of the hyoid apparatus, showed marked degeneration. The soleus muscle in the hindlimb was moderately affected. At this age the mice began to lose body mass and exhibit a hunched posture. At all ages examined, the most severely affected muscle cells were generally located adjacent to bone or at tendinous insertions and occasionally deep within muscle bundles.

Ultrastructure of the Degenerating Muscle Cells

Electron microscopy revealed degenerative changes at the ultrastructural level (Fig. 8). The appearance of amorphous, flocculent, electron-opaque material (FEOM) interspersed between myofibrils was seen in mildly affected cells (Fig. 8, arrows), probably signifying early events in the degenerative process. A mildly affected tongue cell (Fig. 8A) with only a small area of FEOM was adjacent to cells with relatively normal appearances. As the degenerative process progressed in both

**FIGURE 5.** Protection against heat-induced denaturation of proteins in knockout lenses. The soluble fractions of lens homogenates, adjusted to 3.1 mg protein/ml, were heated to 54°C (A) or 63°C (B) in spectrophotometer cuvettes and absorbance at 360 nm monitored. αB knockout lenses (αB<sup>−/−</sup>) had less thermal protective capacity than wild-type (wt), but much more than αA knockout lenses (αA<sup>−/−</sup>).

**FIGURE 6.** Radiographs of an 12-month-old wild-type mouse (top) and a 10-month-old knockout mouse (bottom) clearly illustrate the severe anterior kyphosis and emaciation in aging knockout mice.
tongue (Figs. 8A, 8C, 8D) and soleus (Fig. 8E), the relative proportion of FEOM increased within the cell, with a concomitant loss of myofibrils (black arrowheads) until there were few, if any, myofibrils remaining (Figs. 8C, 8E). Consistent with histologic findings (Fig. 7), large vacuoles (identified by asterisks) were present in the later stages of muscle cell degeneration in both tongue (Fig. 8D) and soleus (Fig. 8E). Degenerative changes were not observed in EDL muscles from the same knockout mice and remained so throughout life, suggesting that HSPB2 has been shown to be less effective than HSPB1 in preventing lens epithelial cells from ultraviolet light or staurosporine-induced apoptosis, and addition of aB does not exhibit markedly increased autokini-

DISCUSSION

aA- and aB-crystallins, members of the family of sHSPs, are both expressed at high levels in the vertebrate lens. Whereas aA is expressed at very low levels in a few nonlenticular tissues, aB is expressed at significant levels in many other tissues and, as the family name implies, is induced by heat and other stresses. A point mutation in the aA gene has been linked to autosomal dominant cataract in humans, and similarly, a point mutation in the aB gene has been linked to a desmin-related myopathy with associated cataract.

Although aA and aB share a high degree of homology and form heteromultimeric aggregates, there appear to be significant differences between these proteins. In vitro, aB has been shown to be less thermodynamically stable, more susceptible to heat-induced conformational changes, a less effective chaperone, and more preferentially dissociated from native a-crystallin aggregates by chaotrophic agents than aA. Unlike aA, aB does not exhibit markedly increased autokinase activity when dissociated to the tetrameric state. In cell culture, aB has been shown to be less effective than aA in preventing lens epithelial cells from ultraviolet light or staurosporine-induced apoptosis, and addition of aB, but not aA, to cultured lens cells has been shown to induce morphologic changes reminiscent of differentiating fiber cells.

We have studied the roles of the two a-crystallins in vivo by removing them by gene targeting in the mouse. Previously, we showed that the absence of aA results in microphthalmia, decreased lens size and weight, cataract formation before the age of 7 weeks, formation of aB-containing cytoplasmic inclusion bodies in lens fiber cells by 4 weeks of age, and a major shift of lenticular aB from the soluble to the insoluble phase. It is therefore possible that a primary function of aA is maintaining the solubility of high concentrations of aB in lens cells. In the present study, we deleted the aB and the closely related HSPB2 genes from the mouse. In contrast to the lens devastation observed in the aA knockout mouse, the lenses of the aB knockout mice were remarkably similar to those of wild-type mice and remained so throughout life, suggesting that aA is more important in lens development and maintenance of lens transparency than aB.

The sizes of aB knockout lenses were the same as those of wild type, and the lens mass of knockout mice was only approximately 7% less than wild type, compared with the approximately 50% mass reduction and 15% reduction axial and equatorial dimensions of the aA knockout lenses. It is
unclear whether this slight mass reduction is due to decreased cell number or a decreased average mass per cell in the knock-out lenses. Despite the small difference in mass, the lenses of αB knockout and wild-type mice appeared very similar in gross morphology and histologically, and although slit lamp examination of the αB knockout lenses often produced slightly more basal light scattering than did wild-type lenses, αB knockout and wild-type lenses dissected and directly compared in dark-field illumination exhibited a similar amount of light scattering. Such discrepancies between slit lamp examinations and examinations of dissected lenses have been previously documented in mice.45 In both slit lamp and dark field, light scattering was far less in lenses of αB knockout mice than those of αA knockout mice.

It has recently been reported that lenses from 129Sv mice do not have the lens-specific beaded filament cytoskeletal proteins cp49 and filensin, as a result of a mutation in the cp49 gene.2 This deficit may be responsible, at least in part, for the basal level of light scattering seen in the lenses of both wild-type and αB knockout mice that are in the mouse 129Sv background. The absence of this cytoskeletal component may also contribute to the vacuoles observed in the outer cortex of lenses fixed with glutaraldehyde followed by formaldehyde (Figs. 3K–P), regardless of whether these vacuoles are true reflections of the in vivo condition or artifacts of tissue preparation resulting from a weakened cytoskeleton.

A key feature of the cataract in αA knockout mice is the presence of cytoplasmic inclusion bodies containing mainly αB,21 indicating that the presence of αA is essential to maintaining the solubility of αB in lens nuclear and inner cortical fiber cells. In the absence of αB, we saw no indication of inclusion bodies containing αA, suggesting that αA is more stable in the lens milieu than αB and does not require its aggregation partner, αB, to remain in solution. This apparent propensity of αB to form inclusion bodies at high concentration is of particular interest, because αB has been found to be a constituent of inclusion bodies in a variety of pathologic conditions46–49 in which αB is upregulated, including Rosenthal fibers in Alexander disease,50 cortical Lewy bodies in Lewy body disease,51 Mallory bodies in alcoholic liver disease,52 and Alzheimer disease plaques and neurofibrillary tangles.49–51

The overall chaperone activity in the αB knockout lens was only mildly diminished compared with that of the αA knockout lens, as demonstrated by protection against heat-induced aggregation of proteins in a lens homogenate supernatant. The thermal protection assay has recently been shown to give results comparable to other types of chaperone assays.41 Contributing to the severely decreased chaperone activity in αA knockout lenses, compared with αB knockout lenses, may be the weaker chaperone activity of αB compared with that of αA44 and the fact that in αA knockout lenses not only is the αA absent, but a sizable portion of the αB is in the insoluble phase and therefore is also absent from the lens homogenate supernatant used for the assay.21 Hyperbaric oxygen treatment of

**Figure 8.** Electron micrographs of tongue and soleus muscle from knockout mice (A, C, D, E) showing various degrees of muscle cell degeneration, and from wild-type mice (B). In one muscle cell (A, top) in the tongue of a knockout mouse, an amorphous, FEOM (A, C, arrows) is interspersed between regularly shaped myofibrils, whereas an adjacent cell (A, bottom) exhibits no such abnormalities. This area of the lower muscle cell is a mitochondria-rich region (mitochondria are dark ovoid bodies). (B) Normal muscle cell architecture is seen in a wild-type mouse soleus muscle. The regular, repeating sarcomeric structure, punctuated by the dark Z line, is readily evident; nothing resembling FEOM was observed in wild-type muscle. (C) In a severely degenerated knockout tongue muscle cell, an isolated, relatively normal-looking myofibril bundle (arrowheads) is surrounded by the FEOM (arrow). (D) In this cross section through a severely affected knockout tongue cell, two vacuoles with irregular edges are present (★). (E) Cell from the hindlimb soleus muscle exhibits a similar pattern of FEOM. At this advanced stage of degeneration, an isolated myofibril bundle (arrowheads) is surrounded by the FEOM, and a large vacuole is evident (★).
mice, used to oxidatively stress lenses in vivo, showed no difference in loss of lens transparency between αB knockout and wild-type mice. This is consistent with the relatively normal chaperone activity in the αB knockout lenses and the relatively normal levels of GSH in lenses of αB knockout mice at all ages.52

Although the lens seems unaffected by the absence of αB and HSPB2, this mutation causes a severe phenotype in older mice that is characterized by hunched posture, loss of body mass after 40 weeks of age, and severe muscle cell degeneration, but in only some muscles. Because both αB and HSPB2 are expressed in skeletal muscle,35,52 and because both genes were disrupted in this knockout mouse, we cannot ascribe this phenotype specifically to either of the proteins; therefore, the muscle phenotype observed may result from the absence of either or both of these closely related sHSPs.

Expression of αB in skeletal muscle is highest in the most oxidative, slow-twitch type I muscle cells, intermediate in fast-twitch type IIA cells, and lowest in the glycolytic, fast-twitch type IIB muscle cells.15,53 consistent with its role as a stress protein, and has been shown to colocalize with desmin at the Z bands.54 The presence of muscle cell degeneration in the cell type I–containing soleus but not in the type II plantaris or EDL suggests, but does not prove, that the oxidative type I muscle cells, which normally would contain the highest levels of αB, are the degenerating cells. Attempts to identify the degenerating muscle cell type by immunostaining with antibodies for fast- or slow-twitch myosin heavy chains were unsuccessful, with the degenerating cells staining for neither type (data not shown). This could have been due to loss of the myosin heavy chain antigen in the severely degenerated muscle cells. Indeed, in a French family with desmin-related myopathy caused by a point mutation in αB,20 the muscles contained cells with a “rubbed-out” appearance when stained for either myosin adenosine triphosphatase (ATPase) activity or oxidative activity, suggesting the loss of myosin in these cells.19 Vacuoles with irregular edges were also observed. These anomalies were found exclusively in type I fibers.19

In the French desmin-related myopathy,19,20,55 a mutant αB protein is present that becomes insoluble and forms dense electron-opaque bodies in affected muscle cells.20 The R120G mutant form of αB in this family was shown in vitro to adopt an irregular structure, have drastically reduced chaperone function, and be found in the insoluble material after chaperone assays.56 This is quite different from the situation in our αB knockout mice, in which the αB was simply absent. In this case, there was no mutant protein with abnormal activity to interfere with cellular processes; thus, we were looking purely at the effect of deletion of the normal protein(s) from the cell. In this regard, some of the anomalies found in our knockout mice are similar to those found in the French family: emaciation, difficulty swallowing, abnormal gait, weakness of muscles in the neck and trunk,19,20 and increased immunohistochemical staining for desmin in severely affected muscle cells (not shown), suggesting that the loss of normal αB function may contribute to both phenotypes.

As stated earlier, we cannot rule out the possibility that the other deleted gene, HSPB2, may be responsible for the observed muscle phenotype. HSPB2, also known as myotonic dystrophy protein kinase binding protein (MKBP), has been shown to activate myotonic dystrophy protein kinase (DMPK) and protect it from heat-induced inactivation53 and is upregulated in myotonic dystrophy. Similar to our knockout mice, mice without DMPK show age-related changes in head and neck muscle fibers57 and late-onset, progressive skeletal myopathy, with elevated muscle fiber degeneration and fibrosis.58 It therefore seems plausible that the absence of HSPB2/MKBP contributes to the observed phenotype by failing to activate DMPK.

Similar to αB, HSPB2 has been localized to the Z lines in skeletal muscle, suggesting that in addition to its role of activating DMPK, it may also play a more direct role in stabilizing the sarcomeric structure.22 However, HSPB2, with its aggregation partner HSPB3, has been shown in muscle cells to form oligomeric complexes that are separate from the complexes formed by αB, HSP25, and HSP20. Thus, although they colocalize at Z lines, the two types of sHSP complexes may have different protective functions there59 and may both be essential for maintenance of sarcomeric structure in oxidative muscle cells.

Because αB is expressed at high levels very early in embryonic heart development, and both αB and HSPB2 are expressed in the fully formed heart, we originally suspected that the gene knockout would cause cardiac problems resulting in embryonic or neonatal lethality. We were surprised to observe that the hearts of knockout mice appeared histologically normal, even at older ages, and functioned sufficiently in mice living in a controlled animal facility environment. However, further studies of cardiac function under a variety of conditions are being conducted by collaborators with cardiology expertise.

In summary, we have shown that αB-crystallin and HSPB2 are not essential for viability or reproduction of the laboratory mouse, αB is not essential for proper lens development, and αB and/or HSPB2 is essential to maintaining integrity of some skeletal muscles. Our data suggesting that αB contributes significantly less to lens development and function than αA agree with recent evidence that αA is a better chaperone than αB,41 that αA can protect lens cells better than αB,39 and that loss of αA leads to a severe decrease in the reducing capacity within the lens, as measured by GSH level, whereas loss of αB has no effect on GSH level.42 This finding also supports the notion that the ancient gene duplication event that gave rise to the two α-crystallin genes, αA evolved to perform critical functions in the lens whereas αB, still highly expressed in the lens, evolved into a more general stress protein. Therefore, although αB may attract broader interest because of its functions in many extraocular tissues such as muscle, αA appears to be the more important protein in lens development and function.

Acknowledgments

The authors thank Mahesh Mankani (National Institute of Dental Research, Bethesda, MD) for performing x-ray analysis of the αB knockout mice; Victor Leverenz and students, Navneet Brar, Valery Heller, Whitney Lakin, Allan Rinkc, and Zhihui Yan (Oakland University), for assistance in the treatment of the mice with hyperbaric oxygen; Yvonne Duglas-Tabor (National Eye Institute) for assistance with two-dimensional gel electrophoresis; and Steven Lee (National Eye Institute) for assistance with mouse genotyping.

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