**Differential Protective Activity of αA- and αB-crystallin in Lens Epithelial Cells**

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αA- and αB-crystallins are molecular chaperones expressed at low levels in lens epithelial cells, and their expression increases dramatically during differentiation to lens fibers. However, the functions of αA- and αB-crystallins in lens epithelial cells have not been studied in detail. In this study, the relative ability of αA- and αB-crystallin, in protecting lens epithelial cells from apoptotic cell death was determined. The introduction of αA-crystallin in the transformed human lens epithelial (HLE) B-3 lens epithelial cell line (which expresses low endogenous levels of αB-crystallin) led to a nearly complete protection of cell death induced by staurosporine, Fas monoclonal antibody, or the cytokine tumor necrosis factor α. To further study the relative protective activities of αA- and αB-crystallins, we created a cell line derived from αA-/αB- double knockout mouse lens epithelia by infecting primary cells with Ad12-SV40 hybrid virus. The transformed cell line αAaBK01 derived from αA/αB double knockout cells was transfected with αA- or αB-crystallin cDNA contained in pcDNA mammalian expression vector. Cells expressing different amounts of either αA-crystallin or αB-crystallin were isolated. The ability of αA- or αB-crystallin to confer protection from apoptotic cell death was determined by annexin labeling and flow cytometry of staurosporine- or UVA-treated cells. The results indicate that the anti-apoptotic activity of αA-crystallin was two to threefold higher than that of αB-crystallin. Our work suggests that comparing the in vitro annexin labeling of lens epithelial cells is an effective way to measure the protective activity of αA- and αB-crystallin. Since the expression of αA-crystallin is largely restricted to the lens, its greater protective effect against apoptosis suggests that it may play a significant role in protecting lens epithelial cells from stress.

α-Crystallin, a member of the small heat shock protein (sHSP) family of molecular chaperones, is an aggregate of two polypeptides, αA- and αB-crystallins, that share 55% amino acid sequence identity. The two ~20-kDa subunits form soluble aggregates with an average molecular mass of 600–800 kDa and can be isolated from lens fiber cells as a heteroaggregate containing αA- and αB-polypeptides in a 3:1 ratio. The α-crys- tallins have dynamic polymeric structures with a hollow interior and undergo inter-aggregate subunit exchange (1, 2).

Initially regarded as lens-specific, the expression of these proteins (especially αB) has since been observed in other tissues, and the two subunits share a ~40% sequence identity with HSP27 (3–5). α-Crystallin is the most abundant soluble protein in the lens and plays an important role in establishing the refractive index of the cytoplasm. However, the extracellular expression of α-crystallins, their autokinase activity, phosphorylation patterns, association with neurodegenerative diseases, and ability to protect against heat shock, argue for a more generalized cellular function of α-crystallins, over and above their role in light refraction (5–9). Like other sHSPs, αA- and αB-polypeptides can act as molecular chaperones in vitro, preventing protein aggregation induced by heat and other stresses (1). Of the two subunits, αA-crystallin expression is considered to be more lens-specific, but only αB-crystallin expression is induced by stress (3–8). However, the in vivo functions and mechanism of chaperone action of the α-crystallin aggregates are largely enigmatic.

Lens growth begins with the division of lens epithelial cells in the germinative zone near the equator. As new cells elongate to form lens fibers, they wrap around the lens periphery and meet at the sutures. Transcripts for αA- and αB-crystallins can be detected in lens epithelial cells at early stages of mouse lens development (10), and a marked increase in αA-crystallin accompanies differentiation (11), but the specific cellular functions of the α-crystallins in the lens epithelium are largely unknown.

Recent work on lens epithelial cells derived from αA- or αB-crystallin knockout mice suggest that they play an important role in regulating cellular growth (9, 12), consistent with the roles of other small heat shock proteins in normal growth and development (13). Because lens epithelial cells grow throughout life, and the lens is exposed to light-induced stress due to its location along the optical axis of the eye, it is likely that mechanisms may be present to protect these cells from a lifetime of exposure to metabolic and environmental stress. αA- and αB-crystallin expression in lens epithelial cells could presumably be helpful in providing protection for these cells.

Recent studies in our laboratory demonstrated that UVA-ase-mediated dUTP nick end labeling; FITC, fluorescein isothiocyanate; PI, propidium iodide; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorting; DAPI, 4′,6-diamidino-2-phenylindole.
radiation, which increases oxidative stress by production of reactive oxygen species and produces cataract in animals, induces lens epithelial cell death, and that the expression of αA-crystallin prevents cell death (9). Because αA-crystallin is largely restricted to the lens, it is important to investigate its role in protecting lens epithelial cells from diverse stress conditions. TNFα is a cytokine that kills cells via apoptosis or necrosis depending on cell type (14). Its mechanism of action involves an increase in reactive oxygen species in cells upon stimulation of TNFα receptors (14). The expression of αB-crystallin and HSP27 prevents TNFα-induced cell death in murine L929 cells, as well as cell death induced by cross-linking of Fas (a member of the TNF and nerve growth family receptors) and staurosporine, a protein kinase C inhibitor (14–16). Proteins of the HSP70 and HSP90 chaperone families also regulate apoptosis (17, 18). The mechanism by which molecular chaperones prevent cell death presumably involves an inhibition of factors that can induce cytchrome c release from mitochondria, and by inhibition of the activation of caspases (19).

The study of physiological roles of the molecular chaperones αA- and αB-crystallin can be assisted with the development of mammalian tissue culture systems in which the cloned crystallin genes can be expressed and the protective activities of the proteins under various stress conditions can be probed. In the present work, we describe cell lines derived from αA- and αB-crystallin knockout mouse lens epithelial cultures in which the two proteins can be re-introduced. These cell lines, as well as transformed human lens epithelial cell lines, allowed us to investigate the relative protective activity of αA- and αB-crystallin in preventing apoptosis of lens epithelial cells induced by staurosporine, TNFα, anti-Fas, and UVA radiation.

**Experimental Procedures**

**Cell Culture and Transfection—**Mouse and human lens epithelial cell cultures were used in this study. The wild-type mouse strain was the 129SvJ mouse. pAKO-127 knockout mice were made by targeted disruption of the αA-crystallin gene (20). pBKO-168 knockout mice were made by the targeted disruption of the αB-crystallin gene. The knock-out vector eliminated the common promoter region of the αB-crystallin and HSPB2 genes, as well as almost all of the coding regions of these two genes (21). Northern blot analysis demonstrated the absence of HSPB2 mRNA in tissues normally expressing this gene (data not shown) confirming disruption of this gene. Mouse clonal cell lines lacking both αA- and αB-crystallins were generated by cross breeding pAKO-127 and pBKO-168 mice (21). Mouse lenses were obtained from 6- to 12-week-old mice. Capsule epithelia of wild-type and knockout lenses were dissected and cultured in 20% fetal bovine serum-minimal essential medium in 24-well tissue culture plates as described earlier (9). Cells were passaged using Trypsin-EDTA and plated in 35-mm plates. In some experiments, cells were grown on sterile glass coverslips to facilitate microscopic examination. Cultures were fed twice weekly. We can differentiate two different kinds of colonies from αB-crystallin knockout mouse lens epithelial cells. When we generated primary cultures of αB knockout lens epithelial cells, we noticed that, in a small proportion of cases, highly proliferative cells are generated. These cells are the subject of another study (12). However, in the current work, we have used only the cells with normal growth.

Extended life span mouse lens epithelial cell lines were also used in this study. These transformed cells were created by infection of primary mouse lens epithelial cultures with Ad12-SV40 hybrid virus using a published procedure (22). To investigate the expression of the HSPB2 gene in transformed mouse lens epithelial cells, we used reverse transcription-polymerase chain reaction with primers specific for the HSPB2 gene (23) and RNA from transformed lens epithelial cells. Wild-type mouse hind limb skeletal muscle RNA was used as a positive control. The results of our study showed that, as in the case of lens tissue and the primary lens epithelial cells, the transformed mouse lens epithelial cell lines did not express the HSPB2 gene (data not shown).

The pCIneoαA expression vector used for human αA-crystallin expression has been described previously (9). The expression vector for human αB-crystallin was made by taking a full-length cDNA clone containing the complete coding sequence of human αB-crystallin from an IMAGE clone (24) (from Genome Systems, Inc., St. Louis, GenBank® accession number N35834). The αB cDNA was cloned into the EcoRI and NotI site of the mammalian expression vector, pCIneo (Promega). The pCIneoαA or pCIneoαB was stably transfected into the transformed double knockout mouse lens epithelial cell line, αAαBKO1, by standard calcium phosphate protocols as described previously (9) using the Promega Profection kit, except that 16 h after transfection cells were given a Me2SO shock according to the manufacturer’s instructions. This procedure increased the transfection efficiency to approximately 40%. 2 days post-transfection, the cells were trypsinized, subcultured 1:3, and Geneticin (0.5 mg/ml) was then added for colony selection. Geneticin-resistant colonies were isolated, and individual colonies were expanded into mass cultures. Each colony is presumably derived from a single cell. Once a mass population of transfected cells was obtained by expansion of the individual colonies, they were used in passage 1 or 2 for assays. Cultures were examined by quantitative immunoblot analysis for the expression of αA- or αB-crystallin (9) and compared with mock transfected cells (cells transfected with vector lacking αA- or αB-crystallin cDNA insert). The expression of αA- or αB-crystallin did not change with passage under these conditions, as determined by immunoblot analysis.

In this study, we investigated stably transfected mouse lens epithelial cell lines expressing between 0.42 and 1.8 mg of human αA-crystallin per μg of cellular protein (isolated from the αAαBKO1 cell lines transfected with pCIneoαA). The cell lines, and concentration of αA-crystallin, given in parenthesis were: cell line MA7 (0.42), MA6 (0.90), MA1 (1.50), and MA2 (1.80). We also investigated clonal cell lines expressing 0.5–2.2 ng of human αB-crystallin per μg of cellular protein (isolated from αAαBKO1 cell lines transfected with pCIneoαB). The cell lines and concentration of αB-crystallin were: cell line MB21 (0.35), MB23 (0.80), MB24 (1.80), and MB25 (2.2). Mock transfected clones were also used from cells that were transfected with the pCIneo vector alone. Mouse clonal cell lines were grown to 80–90% confluence before treatment with staurosporine or UVA radiation as described below. Human lens epithelial cells with extended life span (HLE B-3 cells) have been described previously (22). They were derived from an infant human lens epithelial culture by Ad12-SV40 hybrid virus infection and propagated through at least 11 passages. After passage 11, HLE B-3 cells cease to produce αA-crystallin (as determined by immunoblot analysis) (25). Therefore, αA-crystallin cDNA can be reintroduced into these cells by cDNA transfection, and stably transfected cell lines expressing different amounts of αA-crystallin can be generated. The HLE B-3 cells were cultured in 20% fetal bovine serum-minimal essential medium according to published procedures (22, 25). Transfection of HLE B-3 cells with pCI-neoαA was carried out as described above. The concentration of αA-crystallin in transfected cell lines derived from human lens epithelial clonal cell lines ranged between 0.12 and 2.10 ng of human αA per μg of cellular protein. Human lens epithelial cell lines αA-αBKO1 cell lines transfected with pCI-neoαA (0.12), 405αA (0.45), 504αA (0.70), 809αA (1.40), and 815αA (2.10) were used. Mock transfected cell lines 406, 505, and 511 were also used.

**Induction of Apoptosis—**Cell death was induced by one of the following agents: staurosporine (STP), Fas monoclonal antibody, the cytokine TNFα, or UVA radiation. Optimal conditions were determined for treatment with different apoptosis-inducing agents by varying the concentration and time of incubation. Staurosporine (Sigma) was prepared as a 0.5 mM stock solution and used at 0.5 μM concentration. Cells were treated with staurosporine for 2 h, because readily detectable annexin labeling was observed under these conditions. Human recombinant TNFα (1.08 × 10^6 units/mg, Sigma) was also used to induce apoptosis. We titrated the cells with TNFα and found that the most effective concentration for triggering apoptosis was 2000 units/ml for 3 h. The apoptosis-inducing antibody to Fas (clone DX2 from PharMingen) was used at a concentration of 0.25 μg/ml for 3 h. HLE B-3 cells have been shown to express TNFα receptors and Fas by immunofluorescence and immunoblotting. Cells were treated with UVA radiation at 365 nm at a fluence of 37.8 J/cm² as described previously (9). UVA-irradiated cells were incubated for 4 h after irradiation in normal tissue culture medium supplemented with 10% FCS for annexin labeling below. In some experiments, UVA-treated cells were incubated for varying times in normal tissue culture medium at 37 °C and processed for TUNEL labeling, as described below.

[1] U. P. Andley, Z. Song, R. Kannan, and S. Bassnett, unpublished observations.
Thermal Stress—HLE B-3 cells were grown to confluence in 35-mm tissue culture plates and exposed to mild thermal stress by incubation at 43 °C for 1 h in a water bath. After heat treatment, cells were incubated at 37 °C in a water-saturated air incubator containing 5% CO₂ for times ranging from 0.5 to 24 h, and proteins were analyzed by immunoblot analysis of Triton X-100 for 30 min, and nonspecific binding was blocked by incubation in 10% normal goat serum for 30 min. To visualize the distribution of α-crystallin, cells were incubated overnight at 4 °C in a 1:100 dilution of a monoclonal antibody against bovine α-crystallin (a gift from Dr. Paul Fitzgerald). A lissamine-rhodamine-conjugated goat anti-mouse IgG was used as the secondary antibody. To visualize the organization of F-actin, fixed and permeabilized cells were incubated in a 1:50 dilution in PBS of a fluorescein phalloidin (or Texas Red phalloidin, Molecular Probes Inc.) methanolic stock solution (100 units/ml of methanol). Cells were stained with fluorescein phalloidin for 20 min, washed 3×5 min in PBS, and viewed. Lens epithelial cells were viewed using a Zeiss LSM 410 confocal microscope equipped with an argon-krypton laser.

RESULTS

Human Lens Epithelial Cells—The expression of αA-crystallin in lens epithelial cells has been shown to prevent UV radiation-induced apoptotic cell death. We examined whether αA-crystallin expression also protects cells from various other inducers of apoptotic death. HLE B-3 cells were transfected with pCMneoαA vector carrying human αA-crystallin cDNA or mock transfected with the vector alone. Mock and αA-expressing cells were treated with staurosporine (0.5 μM), incubated for 2 h, and labeled with annexin and propidium iodide (PI). In some experiments, the TUNEL assay was used to detect apoptotic cells. Cells were fixed in 4% paraformaldehyde, pH 7.4, for 30 min, permeabilized for 30 min in 0.1% Triton X-100/PBS at room temperature, and washed with 20 min in PBS (prior to preparing the nuclei) were detected using a TUNEL-labeling reaction according to the manufacturer’s instructions (Roche Molecular Biochemicals). To determine the proportion of apoptotic cells in a culture, cells were co-labeled with 4,6-diamidino-2-phenylindole (DAPI), a DNA-binding fluorophore, which fluoresces in the UV region. TUNEL-positive nuclei and DAPI-labeled nuclei were visualized using a fluorescence microscope (Zeiss). Images were recorded on a cooled, coupled device camera (Photometrix), and the number of TUNEL-positive nuclei and total nuclei were counted using ImageQuanti software. Data analyses were performed with Cell Quest software.

Live cells did not get labeled with either annexin or PI and were identified in the lower left quadrant of the FACS data, as shown in Fig. 1. Annexin-positive but PI-negative cells were interpreted as cells undergoing apoptosis. These cells were identified in the lower right quadrant. Cells that had already died by apoptosis were both annexin-positive and PI-positive, and these cells were identified in the upper right quadrant. PI-negative but annexin-negative cells, identified in the upper left quadrant, were defined as cells that had already died by necrosis. Percentage of protection by αA- or αB-crystallin expression under each stress condition was determined by the ratio of unlabeled, live cells in the mock transfected and αA- or αB-crystallin-expressing clones multiplied by 100.

In some experiments, the TUNEL assay was used to detect apoptotic cells. Cells were fixed in 4% paraformaldehyde, pH 7.4, for 30 min, permeabilized for 30 min in 0.1% Triton X-100/PBS at room temperature, and washed with 20 min in PBS (prior to preparing the nuclei) were detected using a TUNEL-labeling reaction according to the manufacturer’s instructions (Roche Molecular Biochemicals). To determine the proportion of apoptotic cells in a culture, cells were co-labeled with 4,6-diamidino-2-phenylindole (DAPI), a DNA-binding fluorophore, which fluoresces in the UV region. TUNEL-positive nuclei and DAPI-labeled nuclei were visualized using a fluorescence microscope (Zeiss). Images were recorded on a cooled, coupled device camera (Photometrix), and the number of TUNEL-positive nuclei and total nuclei were counted using image analysis software (Metamorph, Universal Imaging Corp.).

Western Blotting—Cells (~10⁶) were washed in PBS, detached with trypsin, and treated with endonuclease (Sigma) for 30 min to degrade DNA. Cells were incubated in lysis buffer containing protease inhibitors leupeptin, pepstatin, and aprotinin (10 μg/ml each), 50 mM Tris-HCl, pH 7.45, 150 mM NaCl, 1% Triton X-100, and 0.5% sodium deoxycholate for 30 min on the ice as described previously (9). Insoluble material was removed by centrifugation, and lysates were run on SDS-polyacrylamide gels. After transfer to Immobilon membranes (Millipore) immunoblot analysis was carried out as described previously (9, 25). The antibody used for immunoblot analysis of αB-crystallin was a rabbit polyclonal antiserum to the 21-amino acid C-terminal peptide of human αB raised in rabbits. The antibody was used at a dilution of 1:1000. To detect HSP27, a polyclonal antibody to human HSP27 (SPA-800) was obtained from StressGen Biotechnologies and used at 1:1000 dilution. The antibody to αA-crystallin was a monoclonal against bovine αA-crystallin (a gift from Dr. Paul Fitzgerald). Immune complexes were detected with HRP-conjugated rabbit IgG, and αA-crystallin was detected with an Alexa488-conjugated goat anti-rabbit IgG. To visualize the distribution of F-actin, fixed and permeabilized cells were incubated in a 1:50 dilution in PBS of a fluorescein phalloidin (or Texas Red phalloidin, Molecular Probes Inc.) methanolic stock solution (100 units/ml of methanol). Cells were stained with fluorescein phalloidin for 20 min, washed 3×5 min in PBS, and viewed. Lens epithelial cells were viewed using a Zeiss LSM 410 confocal microscope equipped with an argon-krypton laser.

Immunoaffluorescence—Western immunoblots gave the level of expression of αA- or αB-crystallin in the cell population as a whole. However, to demonstrate how many cells in the culture expressed the proteins, we also examined the expression of αA- and αB-crystallin in different cells of a culture by immunofluorescence, as described previously (9). Briefly, cells were fixed for 30 min in 4% paraformaldehyde/ PBS and permeabilized in 0.1% Triton X-100 for 30 min, and nonspecific binding was blocked by incubation in 10% normal goat serum for 30 min. To visualize the distribution of αA-crystallin, cells were incubated overnight at 4 °C in a 1:100 dilution of a monoclonal antibody against bovine αA-crystallin (a gift from Dr. Paul Fitzgerald). A lissamine-rhodamine-conjugated goat anti-mouse IgG was used as the secondary antibody. To visualize the distribution of αB-crystallin, cells were incubated overnight at 4 °C in a 1:100 dilution of a polyclonal antibody raised against bovine αB-crystallin (a gift from Dr. Joseph Horwitz). This primary antibody was used, because it gave a very low background in immunocytochemistry with the αB-crystallin—/– mouse lens slices. An Alexa™-conjugated goat anti-rabbit IgG was used as the secondary antibody. To visualize the organization of F-actin, fixed and permeabilized cells were incubated in a 1:50 dilution in PBS of a fluorescein phalloidin (or Texas Red phalloidin, Molecular Probes Inc.) methanolic stock solution (100 units/ml of methanol). Cells were stained with fluorescein phalloidin for 20 min, washed 3×5 min in PBS, and viewed. Lens epithelial cells were viewed using a Zeiss LSM 410 confocal microscope equipped with an argon-krypton laser.

Mock transfected and αA-expressing HLE B-3 cells were also treated with TNFα or Fas monoclonal antibody and labeled with annexin and propidium iodide. Annexin labeling was 18% in the TNFα-treated mock cell line as compared with 3% in the αA-crystallin-expressing clone (data not shown). αA-crystallin expression also decreased annexin labeling in Fas monoclonal antibody-treated cells (36% annexin labeling for mock cells and 5% for αA-expressing cells, data not shown). These results indicate that, by introducing αA-crystallin in cells, apoptosis in response to a variety of agents (as measured by annexin labeling) decreased significantly.

We next analyzed the protective effects of expression of different levels of αA-crystallin in stably transfected HLE B-3 cell
lines exposed to stress-inducing agents. The relative expression of αA-crystallin in transfected cell lines was determined by immunoblot analysis (9) and immunofluorescence. As shown in Fig. 2 (D–F), the individual cells of a given culture expressed similar amounts of the protein, giving a measure of homogeneity in a culture. Fig. 2 (A–C) shows the percentage of protection from cell death induced by staurosporine, TNFα, or anti-Fas in transfected HLE B-3 cell lines and indicates that the relative protection against cell death increased with an increase in expression level of αA-crystallin under all three conditions.
This suggests that αA-crystallin protected against apoptosis induced by a variety of pathways. αA-crystallin may be exerting its protective effect either by activating anti-apoptotic factors (such as Bcl-2), or by preventing the activation of pro-apoptotic factors (such as caspases).

The HLE B-3 lens epithelial cell line used in this study expressed endogenous levels of the small heat shock proteins, αB-crystallin and HSP27, as detected by immunoblotting (Fig. 3). Thermal stress-induced enhancement of expression of these proteins has been demonstrated in a number of non-lens cell lines (5, 6), but it is not known whether HLE B-3 cells are capable of increasing the expression of small heat shock proteins. We investigated the effect of mild thermal stress on the expression of αB-crystallin and HSP27. Exposure of HLE B-3 cells to 43 °C for 1 h, followed by periods of recovery at 37 °C ranging from 1 to 24 h, led to a small increase in HSP27 expression 1 and 4 h after heat shock. HSP27 expression was enhanced considerably between 8 and 18 h after thermal stress (Fig. 3, C and D). In contrast, αB concentration decreased immediately after heat shock and remained lower than control for up to 1 h before increasing to levels above control between 4 and 18 h after thermal stress (Fig. 3, A and B). These results indicate that, as has been reported in other cells, the transformed HLE B-3 lens epithelial cells have the capacity to enhance their expression of heat shock proteins upon exposure to thermal stress. αB-crystallin and HSP27 accumulated at prolonged times after thermal stress. Only αB-crystallin decreased immediately after stress.

Mouse Lens Epithelial Cells—Primary cultures of αB-crystallin+/− mouse lens epithelial cells were compared with wild-type primary cells in their response to UVA radiation-induced cell death. There was no difference between the susceptibility of αB-crystallin+/− and wild-type cells to UVA-induced TUNEL labeling (Fig. 4A). This observation is in striking contrast to the results we reported previously with αA-crystallin+/− lens epithelial cells, which showed a significantly higher level of apoptosis by UVA radiation (9), suggesting that αA-crystallin is more effective in preventing cell death by UVA radiation than is αB-crystallin. Furthermore, when the double knockout primary cells were exposed to UVA radiation,
TUNEL-labeled, and analyzed for extent of labeling, the proportion of TUNEL-labeled cells was similar to that in αA-crystallin knockout cells reported previously. Together, these observations indicate that knocking out αA-crystallin increased UVA-induced apoptotic cell death, whereas knocking out αB-crystallin did not have a significant effect.

To determine whether normal cultures of wild-type mouse lens epithelial cells expressed a uniform level of αA- or αB-crystallin, we examined the expression of the proteins in wild-type lens epithelial cultures by immunofluorescence. As shown in Fig. 4B, the level of expression of αA-crystallin was not uniform in individual cells of a primary culture, with some cells expressing a low level and others expressing higher levels of αA-crystallin. Similar results were obtained when αB-crystallin expression was examined (data not shown).

To investigate the relative protective abilities of αA- and αB-crystallin in more detail, we reasoned that a cell line lacking both αA- and αB-crystallin expression would be useful for transfection with either pCIneoαA or pCIneoαB, to probe the effect of expression of each protein against a null background. To increase the growth potential of the mouse lens epithelial cultures, and enable us to transfect them readily, primary mouse lens epithelial cells of wild-type, αA-crystallin knockout, αB-crystallin knockout, and αA/αB-crystallin double knockout mice were infected with Ad12-SV40 hybrid virus and propagated to make cell lines (Fig. 5A). The expression of αA- and αB-crystallins in transformed cell lines created from wild-type, αA-crystallin knockout, αB-crystallin knockout, and αA/αB double knockout cells was analyzed by immunoblotting (Fig. 5B). As expected, the transformed cell line from the double
knockout mouse lens (αA′αBKO1) was shown to lack the expression of both αA- and αB-crystallin proteins. This cell line was then used for transfection with the pCI-neoA or pCI-neoB containing human αA- or αB-crystallin cDNA, and clones expressing different levels of αA- or αB-crystallin were isolated (Fig. 6). Cells were exposed to staurosporine (0.5 μM) for 2 h, labeled with annexin, and analyzed by FACS analysis. Cells expressing 0.42 ng of αA-crystallin/μg of cellular protein decreased annexin labeling by 25% as compared with mock transfected cells (Fig. 7A). In contrast, cells expressing 0.35 ng of αB-crystallin had the same level of annexin labeling as mock transfected cells. Cells expressing 0.90 ng of αA-crystallin/μg of cellular protein reduced annexin labeling to background levels. In contrast, cells expressing the same level of αB-crystallin did not prevent staurosporine-induced annexin labeling. However, cells expressing 2.2 ng of αB-crystallin were effective in decreasing annexin labeling to background levels. This finding indicates that even a small amount of αA-crystallin expression was protective against cell death. Under the conditions tested, the ability of low concentrations of αA-crystallin to prevent apoptotic cell death was significantly higher than that of αB-crystallin. At higher concentration, αB-crystallin provided the same protective ability as did αA-crystallin.

Additional experiments were carried out to compare the relative ability of αA- and αB-crystallin to protect lens epithelial cells from stress. Mouse lens epithelial cell clones expressing αA- or αB-crystallin were also exposed to UVA radiation. As shown in Fig. 7B, αA-crystallin decreased annexin labeling of UVA-treated cells at lower concentrations than αB-crystallin. However, some differences were noted between the level of protection by αB-crystallin between the two methods used to induce apoptosis. αB-crystallin provided a greater protection against UVA stress than it did against staurosporine-induced apoptosis. This may be attributed to different mechanisms of apoptosis in UVA radiation and staurosporine-induced cell death.

**DISCUSSION**

αA- and αB-crystallins are synthesized as major components of all vertebrate lenses (5). Although both subunits are highly expressed in lens fiber cells, they are also readily detected in lens epithelial cells (10). Because the epithelial cells represent the most metabolically active region of the lens, we focused on cultured lens epithelial cells derived from αA′, αB′, or αA′αB-crystallin knockout mice. A recent study of lens epithelial cells derived from αB-crystallin knockout mice indicated that the loss of αB-crystallin predisposes lens epithelial cells to genomic instability (12), which may explain its broad tissue distribution as well as its overexpression in growing tissues, in diseased states, and under stress conditions (3–5, 26–28). In contrast, lens epithelial cells from αA-crystallin knockout mice were reportedly delayed in growth and were more sensitive to UVA radiation (9). The present results provide new insight into the distinctive cellular functions of αA- and αB-crystallins under physiologically relevant conditions.

One of the main findings of this study is that αA-crystallin expression in lens epithelial cells imparts a greater resistance to various apoptotic inducers than the expression of αB-crystallin. At a given expression level, the anti-apoptotic activity of αA- was significantly higher than that of αB-crystallin. Moreover, αA-crystallin, which has been shown previously to confer resistance to thermal stress and UVA radiation (8, 9), was also found to prevent apoptosis induced by TNFα, staurosporine, and anti-Fas. Lens epithelial cells are proximal to sources of stress and our results suggest that an important in vivo function of αA-crystallin may be to protect these cells from stress-induced apoptosis. Although the mechanism by which αA-crystallin exerts this protective effect is unknown, it may indirectly affect either the activation of anti-apoptotic factors, such as Bcl-2, or prevent the activation of pro-apoptotic factors, such as caspases (19).

Previous work on prevention of stress-induced apoptosis by sHSPs has been restricted to αB-crystallin and HSP25/27 (14–16). Because αA-crystallin expression is largely restricted to the lens tissue, and because lens epithelial cells are continually exposed to sources of environmental stress, we sought to determine the relative protective activity of αA-crystallin and other sHSPs. When primary cultures of wild-type or αB-crystallin knockout mouse lens epithelial cells were irradiated with physiological levels of UVA radiation, no significant increase in apoptosis occurred during the 24-h post-irradiation period. After UVA exposure, the number of apoptotic cells was indistinguishable between wild-type and αB-crystallin knockout cells, suggesting that loss of αB-crystallin did not increase the incidence of apoptosis. In striking contrast, our previous study with UVA-treated αA-crystallin knockout lens epithelial cells had demonstrated that the loss of αA-crystallin resulted in a 40-fold increase in apoptosis (9). We also exposed αA/αB double knockout lens epithelial cultures to UVA radiation. In these double knockout cells, a 40-fold increase in apoptotic cells was observed following UVA radiation. Taken together, the results obtained with primary cultures of αA knockout, αB knockout, and αA/αB double knockout lens epithelial cells indicate that, in the presence or absence of αA-crystallin, the loss of αB-crystallin does not increase apoptosis. This is consistent with the notion that the anti-apoptotic activity of αA-crystallin is higher than that of αB-crystallin. This observation also suggests that HSP25/27 present in lens epithelial cells contributes to maintaining their protection at a certain level.

αA- and αB-crystallin have pleomorphic cellular functions, including the ability to bind cytoskeletal elements (29, 30), bind membranes (24), and translocate to the nucleus (31), although the proteins that interact with αA- and αB-crystallin in the lens epithelium in vivo are unknown. One of the unresolved question in lens research is why are both αA- and αB-crystallin needed in the lens (2)? Recently, it has been shown that a targeted disruption of a mouse αA-crystallin gene induces cataract (20). Interestingly, dense inclusion bodies consisting mainly of αB-crystallin were found in the lens fiber cells of these mice suggesting that αB-crystallin is unstable at high
concentrations in the lens in the absence of its aggregation partner, \( \alpha \)-A-crystallin. Other experiments suggest that mixing of \( \alpha \)-A- and \( \beta \)-B-crystallin increases the stability of the system (2). Although \( \beta \)-B-crystallin is widely distributed in tissues such as muscle, heart, brain, lung, and kidney, \( \alpha \)-A-crystallin is largely restricted to the lens, with low levels detected in the thymus and spleen (3–5). This distinct tissue distribution and the stress indocibility of only \( \alpha \)-B- and not \( \alpha \)-A-crystallin supports the notion that the two proteins may have unique cellular functions in the lens.

Recent in vitro studies demonstrate that the chaperone-like activity of \( \alpha \)-A-crystallins is sensitive to substrate, buffer conditions, and temperature (32–36). Although the activity of \( \beta \)-B-crystallin is relatively insensitive to temperature, a marked temperature dependence of chaperone activity of \( \alpha \)-A-crystallin has been reported (32–36). Using several protein substrates, \( \alpha \)-A- and \( \alpha \)-B-crystallin were found to have similar chaperone activities at physiological temperatures (~37 °C) when inhibition of lactalbumin aggregation was measured, whereas the chaperone-like activity of \( \beta \)-B-crystallin was greater than that of \( \alpha \)-A-crystallin when the substrate was alcohol dehydrogenase or insulin (33). Another factor that may contribute to their different activities is the surface hydrophobicity of the \( \alpha \)-A- and \( \alpha \)-B-crystallins (32–36). However, neither the differences in vitro chaperone activity toward selected substrate proteins, nor the different surface hydrophobicity of \( \alpha \)-A- and \( \alpha \)-B-crystallin are sufficient to explain the greater anti-apoptotic activity of \( \alpha \)-A- over that of \( \alpha \)-B-crystallin found in the present study (32–36). These findings provide a basis for future studies on identification of in vivo substrates of \( \alpha \)-A- and \( \alpha \)-B-crystallin and other factors that determine their in vivo protective activity.

The assessment of relative anti-apoptotic activities of \( \alpha \)-A and \( \alpha \)-B-crystallins in mouse lens epithelial cells using annexin labeling was further supported by our findings in human lens epithelial cells. The extended-life span HLE B-3 cells used for transfection studies did not express endogenous \( \alpha \)-A-crystallin (2, 37). Quantitative immunoblot analysis of HLE B-3 cells (data not shown) indicated that the stoichiometric composition of the \( \alpha \)-A/\( \beta \)-B-crystallin expression in the stably transfected HLE B-3 cells containing >1 ng of \( \alpha \)-A-crystallin/\( \mu \)g of cellular protein is approximately 3:1. Such heteroaggregates were presumably present in \( \alpha \)-A-crystallin-expressing stably transfected HLE B-3 cells, which express endogenous \( \beta \)-B-crystallin. The present results indicate that the expression of \( \alpha \)-A-crystallin, on a background of low \( \beta \)-B-crystallin, is helpful in enhancing resistance of lens epithelial cells to apoptosis induced by a variety of agents and supports the idea that \( \alpha \)-A-crystallin is an important anti-apoptotic protein in lens epithelial cells.

In summary, the present studies indicate that \( \alpha \)-A-crystallin has a higher capacity than \( \beta \)-B-crystallin to prevent lens epithelial cell death. This may indicate that the threshold concentration of \( \alpha \)-A-crystallin necessary for protection against stress-induced apoptosis in the lens epithelium is less than that of \( \beta \)-B-crystallin. Because their expression in the lens epithelium is at much lower levels than in the fiber cells, it remains to be determined whether the 2- to 3-fold difference in protective activity is of significance for their function in the in vivo lens epithelium. The current work suggests that comparing the annexin labeling of lens epithelial cells is an effective way to measure the protective activity of \( \alpha \)-A- and \( \beta \)-B-crystallin under physiologically relevant conditions. Taken together with our previous study (12), which demonstrated that the lack of \( \beta \)-B-crystallin predisposes cultured lens epithelial cells to genomic instability, these studies provide a basis for further investigating the distinct physiological functions of \( \alpha \)-A- and \( \beta \)-B-crystallins in lens epithelial function.

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REFERENCES
1. Horwitz, J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10449–10453
2. Horwitz, J., Bova, M. P., Ding, L.-L., Haley, D. A., and Stewart, P. L. (1999) Eye 13, 403–407
3. Bhat, S. P., and Nagineni, C. N. (1989) Biochem. Cell Biol. 158, 319–325
4. Dubin, R. A., Wawrousek, E. F., and Piatigorsky J. (1989) Mol. Cell. Biol. 9, 1083–1091

FIG. 7. Differential protection by \( \alpha \)-A- and \( \beta \)-B-crystallin against staurosporine or UVA-induced apoptosis. A, mouse lens epithelial clonal cell lines expressing different concentrations of \( \alpha \)-A- or \( \beta \)-B-crystallin were treated with 0.5 \( \mu \)M staurosporine for 2 h, labeled with annexin and PI, and analyzed by FACS. B, mouse lens epithelial clonal cell lines expressing different concentrations of \( \alpha \)-A- or \( \beta \)-B-crystallin were exposed to 37.8 J/cm² UVA radiation (365 nm) and incubated in normal medium for 4 h. Cells were labeled with annexin and PI and analyzed by FACS. The number of \( \alpha \)-A- or \( \beta \)-B-crystallin expressing cells was the same (10⁶) under each condition. Note that, for both treatments, \( \alpha \)-A-crystallin provided significantly better protection than did \( \beta \)-B-crystallin at low expression levels.
1. Sax, C. M., and Piatigorsky, J. (1994) *Adv. Enzymol. Relat. Area Mol. Biol.* **69**, 155–201
2. Klemenz, R., Frohli, E., Steiger, R. H., Schafer, R., and Aoyama (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 3652–3656
3. Kantorow, M., and Piatigorsky, J. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 3112–3116
4. Van den Ijssel, P. L. R. A., Overkamp, P., Knauf, U., Gaestel, M., and de Jong, W. W. (1994) *FEBS Lett.* **335**, 54–56
5. Andley, U. P., Song, Z., Wawrousek, E. F., and Bassnett, S. (1998) *J. Biol. Chem.* **273**, 31252–31261
6. Robinson, M. L., and Overbeek, P. A. (1996) *Invest. Ophthalmol. Vis. Sci.* **37**, 2276–2284
7. Vermorken, A. J. M., Honda, T., van de Ven, W. J., and Bloemendal, H. (1978) *J. Cell Biol.* **76**, 175–183
8. Andley, U. P., Song, Z., Wawrousek, E. F., Brady, J. P., Bassnett, S., and Fleming, T. P. (2000) *FASEB J.*, in press
9. Muchowski, P. J., Valdez, M. M., and Clark, J. I. (1999) *Invest. Ophthalmol. Vis. Sci.* **40**, 951–956
10. Nicholl, I. D., and Quinlan, R. A. (1994) *EMBO J.* **13**, 945–953
11. Bhat, S. P., Hale, I. L., Matsumoto, B., and Elghanayan, D. (1999) *Eur. J. Cell Biol.* **78**, 143–150
12. Datta, S. A., and Rao, Ch. M. (1999) *J. Biol. Chem.* **274**, 34773–34778
13. Reddy, G. B., Das, K. P., Petrash, J. M., and Surewicz, W. K. (2000) *J. Biol. Chem.* **275**, 4565–4570
14. Sun, T.-X., and Liang, J. J. N. (1998) *J. Biol. Chem.* **273**, 286–290
15. Mehl, P., Mehl, A., Guillet, D., Previle, X., and Arrigo, A.-P. (1995) *J. Cell Biochem.* **58**, 248–259
16. Mehl, P., Kretz-Reny, C., Previle, X., and Arrigo, A.-P. (1996) *EMBO J.* **15**, 2695–2706
17. Mehl, P., Schulze-Ostoff, K., and Arrigo, A.-P. (1996) *J. Biol. Chem.* **271**, 16510–16514
18. Mesner, D. D., Caron, A. W., Bourget, L., and Denis-Larose, C. (1997) *Mol. Cell. Biochem.* **17**, 5317–5327
19. Gallo, L., Richardson, A. J., Latchman, D. S., and Katz, D. R. (1996) *J. Immunol.* **157**, 4109–4118
20. Brady, J. P., Garland, D., Douglas-Tabor, Y., Robinson, W. G., Jr, Groome, A., and Wawrousek, E. F. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 884–889
21. Wawrousek, E. F., and Brady, J. P. (1998) *Invest. Ophthalmol. Vis. Sci.* **39**, S523
22. Andley, U. P., Rhim, J. S., Chylack, L. T., and Fleming, T. P. (1994) *Invest. Ophthalmol. Vis. Sci.* **35**, 3094–3102
23. Iwaki, A., Nagano, T., Nakagawa, M., Iwaki, T., and Fukumaki, Y. (1997) *Genomics* **45**, 386–394
24. Cobb, B. A., and Petrash, J. M. (2000) *J. Biol. Chem.* **275**, 6664–6672
25. Fleming, T. P., Song, Z., and Andley, U. P. (1998) *Invest. Ophthalmol. Vis. Sci.* **39**, 1387–1398
26. van Noort, J. M., van Sechel, A. C., Bajramovic, J. J., el Ouagmiri, M., Polman, C. H., Lassman, H., and Ravid, R. (1995) *Nature* **375**, 798–801
27. Iwaki, T., Kume-Iwaki, A., Liem, R. K. H., and Goldman, J. E. (1989) *Cell* **57**, 71–78
28. Klemenz, R., Frohli, E., Aoyama, A., Hoffman, S., Simpson, R. J., Moritz, R. L., and Schafer, R. (1991) *Mol. Cell. Biol.* **11**, 803–812
29. Arrigo, A.-P., and Landry, J. (1994) in *The Biology of Heat Shock Proteins and Molecular Chaperones* (Morimoto, R. I., Tissieres, A., and Georgopoulos, C., eds), pp. 335–373, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
30. Nicholl, I. D., and Quinlan, R. A. (1994) *EMBO J.* **13**, 945–953
31. Bhat, S. P., Hale, I. L., Matsumoto, B., and Elghanayan, D. (1999) *Eur. J. Cell Biol.* **78**, 143–150
32. Datta, S. A., and Rao, Ch. M. (1999) *J. Biol. Chem.* **274**, 34773–34778
33. Reddy, G. B., Das, K. P., Petrash, J. M., and Surewicz, W. K. (2000) *J. Biol. Chem.* **275**, 4565–4570
34. Sun, T.-X., and Liang, J. J. N. (1998) *J. Biol. Chem.* **273**, 286–290
35. Horwitz, J., Huang, Q. L., Ding, L., and Bova, M. P. (1998) *Methods Enzymol.* **290**, 365–383
36. van Boekel, M. A. M., de Lange, F., de Grijp, W., and de Jong, W. W. (1999) *Biochim. Biophys. Acta* **1434**, 114–123
37. Sun, T.-X., and Liang, J. J. N. (1998) *J. Biol. Chem.* **273**, 286–290