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Interactions between Small Heat Shock Protein α-Crystallin and Galectin-Related Interfiber Protein (GRIFIN) in the Ocular Lens[†]

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Abstract

As a member of the small heat shock protein superfamily, α-crystallin has a chaperone-like ability to recognize and bind denatured or unfolded proteins and prevent their aggregation. Recent studies suggest that α-crystallin may also interact with a variety of proteins under native conditions in vitro. To identify potential binding partners for α-crystallin in the intact ocular lens, we conducted cross-linking studies in transgenic mouse lenses designed for overexpression of His-tagged human αA-crystallin. Interacting proteins were copurified with the epitope-tagged crystallin complexes and were identified by tandem mass spectrometry. This approach identified GRIFIN (galectin-related interfiber protein) as a novel binding partner. Consistent with results from cross-linking, GRIFIN subunits copurified with α-crystallin complexes during size exclusion chromatography of nontransgenic mouse lens extracts prepared without chemical cross-linking. Equilibrium binding to GRIFIN was studied using native α-crystallin isolated from calf lenses as well as oligomeric complexes reconstituted from recombinant αA- and αB-crystallin subunits. Calf lens α-crystallin binds GRIFIN with relatively high affinity (K_d=6.5 ± 0.8 μM) at a stoichiometry of 0.25 ± 0.01 GRIFIN monomer/α-crystallin subunit. The binding interaction between α-crystallin and GRIFIN is enhanced up to 5-fold in the presence of 3 mM ATP. These binding data support the hypothesis that GRIFIN is a novel binding partner of α-crystallin in the lens.

The mammalian lens contains a very high concentration of soluble protein, estimated to be approximately 350 mg/mL, to provide refractive power. The majority of lens proteins belong to one of three major groups, which are designated α-, β-, and γ-crystallins (1). The α-crystallins, which constitute ~30% of lens protein, are heteromeric oligomers composed of two gene products, αA and αB, present in a 3:1 molar ratio in human lenses. The two ~20 kDa gene products assemble into oligomers with an average size of 600 kDa, indicating each complex contains approximately 30 monomers (2). The quaternary structure of α-crystallin is very dynamic due to the ability of αA- and αB-crystallin subunits to exchange between complexes in a temperature and time-dependent manner (3, 4).

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Like other members of the small heat shock protein (sHSP)[1] family, α-crystallin has a chaperone-like activity defined by the ability to bind denatured or unstable proteins and prevent their aggregation. While sHSPs have minimal, if any, capacity to actively refold proteins, current evidence from in vitro studies suggests that they can transfer captured protein substrates to conventional chaperones for ATP-dependent refolding (5, 6). Mice with targeted disruption of the αA gene develop early cataracts caused by accumulation of light scattering protein aggregates (7), consistent with the view that a critical role for α-crystallin in the lens is maintenance of transparency. However, the mechanisms by which α-crystallin recognizes unstable and/or unfolded proteins are still unknown.

Recent studies demonstrate that α-crystallin can interact with a wide variety of proteins involved with signaling and cytoskeletal structure (8). α-Crystallin interacts with various components of the cytoskeleton, including actin, vimentin, CP49, and filensin, but the effect this confers is variable (9–12). α-Crystallin stabilizes actin filaments both in vivo and in vitro when subjected to stresses, including heat and cytochalasin D (11, 12). However, the interaction with type III intermediate filament monomers prevents their assembly (10). α-Crystallin also associates with plasma membranes from lens fiber cells, and the total amount bound increases with age, diabetes, and cataract (13, 14). In vitro analysis of this binding suggests the interaction is hydrophobic and not dependent on a specific type of lipid (15). Beyond these insoluble cellular structures, the soluble protein interactions of α-crystallin are largely unknown.

In vitro data suggest that intermolecular interactions occur between α-crystallin and a variety of signaling proteins and metabolic enzymes under native conditions (8, 16); however, the physiological importance is difficult to gauge since most of the identified proteins are expressed in the lens in small amounts, if at all (8). Judging from in vitro studies, substrate binding is significantly enhanced by ATP (16), although little is known about the effect this may have on its ability to bind and/or refold captured proteins (16–18).

The goal of our study is to identify proteins that interact with α-crystallin in the context of the intact lens. As in previous studies which used epitope tagging to selectively enrich proteins that interact with HSP16.6 in the cyanobacterium *Synechocystis* sp. PCC 6803 (19), we have utilized a transgenic mouse model designed for lens-specific expression of His-tagged human αA-crystallin (20). Protein–protein interactions in the intact lens were fixed by a brief treatment with a cross-linking agent. α-Crystallin complexes that contain the bait transgene product were isolated by immobilized metal affinity chromatography (IMAC) for analysis by liquid chromatography–tandem mass spectrometry (LC–MS/MS). As expected, the majority of interacting proteins captured by this approach were crystallins. Proteomic analysis of crystallin complexes isolated from transgenic lenses identified peptides derived from galectin-related interfiber protein (GRIFIN). Binding of GRIFIN by α-crystallin was confirmed using a filter-based binding assay. Dissociation constants and effects of ATP on binding are consistent with physiologically relevant interactions between α-crystallin and GRIFIN.

MATERIALS AND METHODS

Cross-Linking with Intact Lenses

Lenses were dissected from 4–6-week-old mice and briefly rinsed in PBS before being treated with cross-linking agents. For cross-linking with DSS (disuccinimidyl suberate),

[1]Abbreviations: DEAE, diethylaminoethyl; GRIFIN, galectin-related interfiber protein; sHSP, small heat shock protein; IMAC, immobilized metal affinity chromatography; LC–MS/MS, liquid chromatography–tandem mass spectrometry; SEC, size-exclusion chromatography; PEI, polyethyleneimine.

lenses were placed in cross-linking buffer [20 mM $NaPO_4$ (pH 7) and 150 mM NaCl] and treated with 0.5–5 mM DSS for 30 min at room temperature according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL). The reaction was quenched by the addition of 1 M Tris-HCl (pH 7.4) to a final concentration of 20 mM. Lens homogenates were prepared in His-trap binding buffer [10mM imidazole, 6M guanidine-HCl, 20mM Tris, and 500mM NaCl (pH 7.4)]. Alternatively, lenses were incubated with 1% paraformaldehyde for the indicated times at 37 °C. Treated lenses were washed with PBS, and lens homogenates were prepared in His-trap binding buffer containing ~1 mM glycine. In all cases, a shift to a lower relative mobility of proteins on SDS–PAGE analysis was taken as evidence of cross-linking. Proteins were visualized by either Coo-massie Blue staining or Western blot analysis with specific antisera.

Size Exclusion Chromatography

Chromatography of lens homogenates and recombinant proteins was carried out using Superdex 200 (GE Healthcare Biosciences) operated in a 16 cm × 60 cm column at a flow rate of 1 mL/min. Protein elution was monitored with an in-line UV absorbance detector, and fractions were collected throughout. Lens homogenates were prepared in an extraction buffer [10 mM Tris (pH 6.8), 100 mM NaCl, 0.5 mM EDTA, and 1 mM DTT] using 10 mL/g of tissue. Column fractions were analyzed by Western blotting using antisera against α-crystallin (generously provided by U. Andley) or a His-tag epitope (Novagen, San Diego, CA). His-tagged proteins in chromatography fractions were also measured by an ELISA in a microtiter plate format.

Western Blotting

Proteins resolved by SDS–PAGE were electroblotted to PVDF membranes and probed with specific antisera. Immune complexes were identified by incubating blots with horseradish peroxidase-conjugated secondary antibodies and visualized by enhanced chemifluorescence (ECL*plus*, GE Healthcare Biosciences, Piscataway, NJ), and spot density was calculated using ImageQuant (GE Healthcare Biosciences).

Immobilized Metal Affinity Chromatography

His-Trap FF Ni Sepharose Resin (GE Healthcare Biosciences) was equilibrated with binding buffer [10 mM imidazole, 6 M guanidine-HCl, 20mM Tris, and 500mM NaCl (pH 7.4)] on an AKTA FPLC system (GE Healthcare Biosciences). Samples were dialyzed into binding buffer and were filtered (0.2 μm) before being injected onto the column. After the His-Trap resin had been washed with twice the sample volume of binding buffer, proteins were eluted in a 0 to 0.5 M imidazole gradient contained in 20 mL of binding buffer. The eluate was monitored by in-line A_{280} measurements and immunoblotting as described previously.

Mass Spectrometry Analysis and Protein Identification

Purified α-crystallin and substrate complexes were concentrated and dialyzed into 10 mM Tris-HCl (pH 8.0). Samples were acid precipitated and resolubilized in 8 M urea, 100 mM Tris, and 1% Rapigest (Waters, Milford, MA). After reduction with 5 mM TCEP [tris(2-carboxyethyl) phosphine], alkylation was carried out with 10 mM iodoacetamide. Samples were then sequentially digested with LysC (Roche) and trypsin (Sigma). Digested protein fragments were acidified with 5% formic acid and injected for LC–MS/MS on a capillary reverse phase HPLC–MS/MS system using an electrospray-quadrupole time-of-flight mass spectrometer (Q-STAR XL, Applied Biosystems, Foster City, CA) interfaced with a liquid chromatograph (Eksigent, Livermore, CA). Database searching was performed using MASCOT (Matrix Science, Oxford, U.K.) against the NCBI database. Protein

identifications, based on MASCOT scores of > 40, were confirmed by interpretation of the fragmentation spectra.

Cloning and Purification of GRIFIN

A full-length mouse GRIFIN cDNA clone was obtained as an IMAGE clone from Open Biosystems (Huntsville, AL). QuikChange (Qiagen) site-directed mutagenesis was conducted to create a 5′ *Nde*I site (primer pair, 5′ CTTAGTGGCTGAGG-CATAGACACTGCAGTTCGAAGC 3′ and complement) to the GRIFIN cDNA. The open reading frame for GRIFIN was then cloned into a pET-23d(+) vector (Novagen) for overexpression in *Escherichia coli* strain BL21. Purification of GRIFIN was completed essentially as described previously (21), with a few minor changes. After cell lysis and PEI precipitation, the lysate was dialyzed into 50mM Tris (pH 8.5) and loaded onto a DEAE (Sigma, St. Louis, MO) column pre-equilibrated with the same buffer. The column was washed in equilibration buffer, and the protein was eluted with a 0 to 500mM NaCl gradient in equilibration buffer. Elution of the protein was monitored by A_{280} readings, and SDS–PAGE reconfirmed the location and purity of the protein. The pooled protein following the first step was concentrated and loaded onto a G-75 (Bio-Rad) gel filtration column using PBS for protein elution at 1 mL/min. Elution of the protein was followed by A_{280} readings. Western blotting with antisera to mouse GRIFIN (kindly provided by R. Lang, Children's Research Foundation, Cincinnati, OH) was used to confirm the identity of the purified protein. GRIFIN was estimated to be essentially pure judging by the absence of contaminating protein bands following SDS–PAGE and Coo-massie Blue staining of the purified recombinant material.

Purification of Recombinant Human αA- and αB-Crystallins and Bovine and Mouse Lens α_L-Crystallins

For recombinant protein subunits, coding regions of each gene were inserted into pET-23d (Novagen) and over-expression and purification were performed essentially as previously described (22). Native bovine or mouse α_L-crystallin was purified in two sequential gel filtration steps from homogenized bovine cortical lens fiber cells or whole mouse lenses, respectively. A Sephacryl 500 (GE Healthcare Biosciences) gel filtration column (16 cm × 60 cm) was used to separate the α-crystallin fraction (M_r ~ 350000–1000000) from the rest of the soluble lens proteins. Further resolution of the α-crystallin pool was then accomplished using Superdex 200, which separated the high- and low-molecular weight complexes of α-crystallin, essentially as described previously (23). Fractions corresponding to approximately M_r = 350000–650000 were pooled as α_L-crystallin. The essential purity of this material was confirmed by the sole presence of protein bands corresponding to αA- and αB-crystallin subunits following SDS–PAGE. The 3:1 αA:αB hetero-oligomer was formed from purified recombinant crystallins by incubating the appropriate molar ratio of the two subunits in PBS for 18 h at 37 °C (3).

Protein Binding Assays

A membrane filtration assay was used to monitor the binding of α-crystallin to GRIFIN or γ-crystallin essentially as described previously (16). Concentrations shown for crystallins and GRIFIN are calculated on the basis of monomer molecular weights. Binding mixtures containing the indicated concentrations of α-crystallin in 3–30 μM GRIFIN or γ-crystallin in 50 mM potassium phosphate buffer (pH 7.2) and 3 mM ATP were incubated for 2 h at 37 °C. Solutions were cooled to 23 °C for 1 h and then passed through a 100 kDa molecular mass cutoff membrane filter (Microcon, Millipore, Billerica, MA) by centrifugation at 15000 *g*. The amount of free GRIFIN and total GRIFIN was determined with the BCA Protein Assay Kit (Pierce Biotechnology). In preliminary studies to validate the assay, we

determined that α-crystallin subunits were completely retained by the membrane filter. Therefore, filtrate solutions contained only unbound substrate proteins (data not shown).

RESULTS

Transgenic Lens Model System

To examine interactions between human αA-crystallin and native lens proteins, we utilized the CRYAA^{WT} strain of transgenic mouse designed for expression of human αA-crystallin in lens fiber cells (20). The transgene construct was designed to encode a seven-residue His tag at the amino terminus of human αA-crystallin (7×His-αA). The amino-terminal His tag, which has a minimal effect on chaperone-like activity, provides a convenient epitope for affinity purification and antibody staining (24). To determine whether 7×His-αA subunits exchange into hetero-oligomeric complexes containing endogenous α-crystallins in the context of the transgenic mouse lens, we examined the size distribution of oligomeric complexes by size exclusion chromatography (SEC). In contrast to purified recombinant 7×His-αA, which eluted as a well-defined peak corresponding to > 669 kDa, the 7×His-αA subunits in homogenates from transgenic lenses were detected in a broad peak overlapping that of endogenous mouse α-crystallins in the relative molecular mass range of 350–650 kDa (Figure 1a). The 7×His-αA signal was low or essentially undetectable from fractions that correspond to the high-molecular mass range typical of α-crystallin chaperone complexes, or among pooled fractions that correspond to the β- and γ-crystallins (Figure 1b). These results indicate that the human αA-crystallin subunits are integrated into the native like population of α-crystallin oligomeric complexes in the transgenic mouse lens. We reasoned that transgene-derived αA-crystallin subunits containing an affinity epitope would provide a mechanism for selectively retrieving substrate proteins that interact with α-crystallin in the context of the intact lens. Because human and mouse αA-crystallins are structurally and functionally similar by many criteria, we constructed the transgene using the sequence of human αA-crystallin. This strategy enabled us to to distinguish peptides derived from human and mouse α-crystallins during proteomic analysis.

In Situ Protein Cross-Linking

We used in situ cross-linking to capture proteins that interact with the sHSP α-crystallin in the context of the intact lens. Cross-linking provided a convenient method for stabilizing weak and/or transient interactions. In preliminary trials, we observed no evidence of protein cross-linking following treatment of mouse lenses (with or without capsule epithelium) for up to 12 h with up to 5 mM DSS (disuccinimidyl suberate), a membrane permeable crosslinking agent (data not shown). In contrast, distinct protein cross-linking, demonstrated by laddering of α-crystallin subunits, was observed when lenses were treated with 1% formaldehyde for 30–90 min (Figure 2). After 60 min, a significant amount of cross-linked protein became insoluble, accounting for the decreased amount present on the Western blot.

Following formaldehyde treatment, lenses were homogenized in the presence of excess glycine to quench nonspecific cross-linking. Because of the stability of formaldehyde cross-links, all homogenization buffers contained 6 M guanidine. We reasoned that nonspecific interactions that could occur between sHSPs and destabilized proteins produced during tissue disruption would be minimized in the presence of the denaturant. Protein complexes containing the 7×His-αA transgenic protein were isolated by His-Trap Ni Sepharose affinity chromatography. Immunoblots using an anti-His-tag primary antibody confirmed the presence of 7×His-αA in column fractions eluted by imidazole, as evidenced by a distinct laddering pattern (Figure 2). As a control, lenses from age-matched nontransgenic mice were subjected to formaldehyde treatment in the same manner as transgenic lenses. Affinity chromatography was carried out exactly as for proteins from transgenic lens, and the

corresponding imidazole elution fractions were pooled for further analysis. The amount of protein recovered following affinity chromatography using nontransgenic lenses was approximately 8-fold smaller than the amount recovered from transgenic lenses (0.15 and 1.2 mg, respectively).

Identification of Copurified Proteins by Mass Spectrometry

Proteins eluted from the His-trap column were sequentially digested with LysC and trypsin and the resulting peptides separated by liquid chromatography before tandem mass spectrometry analysis (MS/MS). MS/MS analysis with database searching identified an array of proteins found in the purified α-crystallin complexes (Table 1). The equivalent protein pool for the nontransgenic control lens homogenate was processed in the exact same manner to account for any proteins that were recovered under identical column conditions (Table 2).

Thirty-eight proteins were identified in the transgenic mouse α-crystallin complex pool (Table 1). Besides the bait protein (His-tagged human αA-crystallin) and mouse α-crystallins, most of the other proteins were identified as members of the $\beta\gamma$-crystallin superfamily. The cytoskeletal proteins vimentin and actin, which are reported to interact with the sHSP subunits, were also identified (9, 10, 12). The highly abundant crystallins also made up a large number of proteins identified in the nontransgenic control sample. A variety of non-mouse proteins, including human keratins, bovine albumin, and bovine lactoglobulin, were identified in samples purified from transgenic and/or nontransgenic lenses. Such proteins are frequently identified as environmental contaminants in liquid chromatography/MS proteomic studies and were therefore not included in Tables 1 and 2.

Besides human αA-crystallin, a major protein that was uniquely associated with complexes purified from transgenic lenses was identified as GRIFIN (galectin-related interfiber protein). The MS/MS spectra at accurate mass for the doubly charged peptides corresponding to GRIFIN are shown in Figure 3. Peptide fragmentation was consistent with the amino acid sequences of FSSATVVGNAFQGGR and LAQVELAK, which unambiguously matched amino acids 51–65 and 127–134 of GRIFIN with MASCOT scores of 66 and 63, respectively. GRIFIN was identified in each of two independent cross-linking experiments with transgenic lenses. In each case, the identification was made with two peptide fragments obtained from MS/MS analysis of protein complexes isolated by affinity chromatography. Peptide fragments corresponding to GRIFIN were not present in nontransgenic control samples isolated from two different cross-linking experiments.

Copurification of GRIFIN and α-Crystallin in a Non-Cross-Linked Lens

To exclude the possibility that cross-linking induced the interaction between GRIFIN and α-crystallin, we examined the distribution of GRIFIN among elution fractions from size exclusion chromatography of homogenates produced from wild-type (nontransgenic) mouse lenses not subjected to treatment with formaldehyde cross-linking agent. Fractions collected across the elution profile were analyzed for the presence of GRIFIN by Western blot, and the relative densities were calculated [Figure 4 (●)]. The majority of GRIFIN eluted at a volume corresponding to approximately 35 kDa, as expected for the homodimer thought to exist in vivo (21). However, a substantial amount of GRIFIN co-eluted in fractions containing α-crystallin in the broad peak around 600 kDa. For the sake of comparison, we investigated the elution behavior of γ-crystallin, a protein known to bind to α-crystallin in vivo and found to have a K_d of ~3 μM by similar in vitro studies (16). The elution profile of γ-crystallin was strikingly similar, with a small proportion co-eluting with the α-crystallin peak and the majority eluting at a volume consistent of its ~21 kDa molecular mass [Figure

4 (■)]. These results are consistent with the hypothesis that GRIFIN interacts with α-crystallin in a manner that is not dependent on chemical cross-linking.

Interactions between GRIFIN and α-Crystallin

We used an in vitro membrane filtration assay to directly test for binding of GRIFIN to α-crystallin lacking a histidine tag. After incubation for 2 h at 37 °C, binding mixtures containing GRIFIN and α-crystallin were centrifuged through a 100 kDa molecular mass filter. Complexes formed between GRIFIN and α-crystallin remained in the retentate, while unbound GRIFIN passed through the filter. Analysis of the filtrate allowed the amount of free GRIFIN to be quantified. In initial studies, we assessed binding of GRIFIN to α_L-crystallin purified from bovine lens cortex (23). As shown in Figure 5, a relatively small amount of GRIFIN was bound by α-crystallin in the absence of ATP. However, GRIFIN binding was enhanced more than 2-fold when ATP was added up to a concentration of 3mM. In addition, we used recombinant αA- and αB-crystallin subunits either alone or after reconstitution into hetero-oligomeric complexes. As with native α-crystallin, all recombinant forms demonstrated GRIFIN binding that was markedly enhanced in the presence of ATP (Figure 5). Under these conditions, no apparent GRIFIN binding was observed when α-crystallin was either absent or replaced with BSA (data not shown), ruling out the possibility that ATP induces aggregation and/or nonspecific interactions of GRIFIN with the membrane filter. Rather, the results demonstrate that physiological concentrations of ATP enhance interactions between GRIFIN and α-crystallin.

Binding isotherms were created by incubating various amount of GRIFIN with the purified bovine α_L-crystallin at 12.5 μM with 3 mM ATP. Due to the hyperbolic nature of the plot of bound GRIFIN (*xb*) versus free ligand (*xf*) (Figure 6a), a model of independent and identical binding sites was used for the nonlinear least-squares fitting of the binding data. Values for the maximum concentration of GRIFIN bound (B_{max}) and the dissociation constant (K_d) were determined by fitting the curve to eq 1.

$$xb = \frac{B_{max} \times xf}{K_d + xf} \quad (1)$$

By dividing the B_{max} value of 3.13 ± 0.17 μM by the total concentration of α-crystallin, we determined the stoichiometry (*n*) in terms of GRIFIN and monomeric concentrations of crystallin to be 0.25 ± 0.01. The K_d was found to be 6.5 ± 0.8 μM, similar to values previously seen for the interaction of α-crystallin with γ-crystallin (16). The data were also analyzed with a linear Scatchard plot (Figure 6b) by rearranging eq 1 assuming independent and identical sites.

$$\frac{xb}{xf} = \frac{1}{K_d}(B_{max} - xb) \quad (2)$$

When the data were plotted with ratio of bound to free GRIFIN versus bound GRIFIN, the K_d and stoichiometry were found to be 6.13 μM and 0.24, respectively. These values, determined with Scatchard analysis, were within error of those from the isotherm fitting results.

To reconstitute a homologous binding system (mouse crystallin and GRIFIN), we carried out filtration binding assays using mouse α_L-crystallin in place of bovine α_L-crystallin. Binding isotherms (Figure 6c) were similar between bovine and mouse crystallins, with the mouse α_L-crystallin exhibiting a slightly higher K_d (13.6 ± 5.3 μM) and a slightly higher

B_{max} (5.4 ± 0.9 μM). The stoichiometry is ~0.4 ± 0.08 GRIFIN subunit per α-crystallin monomer. The values for K_d and stoichiometry determined by Scatchard analysis (Figure 6d) were 9.4 μM and 0.34, respectively, and were within error of those from the isotherm fitting results.

DISCUSSION

Similar to other members of the sHSP family, α-crystallin has a chaperone-like ability to bind and prevent aggregation of destabilized proteins (8, 16, 25–28). Chaperone- like binding has been demonstrated with a wide variety of protein substrates, suggesting that the intermolecular interactions are not dependent on specific structural motifs presented by substrate proteins. Rather, the binding is due to the ability of α-crystallin to sense or detect denatured or unfolded proteins (29, 30). A variety of lens structural proteins have been demonstrated to interact with α-crystallin on the basis of equilibrium dialysis using lens protein extracts (31) or mammalian tissue culture models (32, 33), but there are no reports from a global search for binding partners captured from interactions with an unfractionated collection of lens proteins. A short-coming of targeted binding studies is that none of these systems adequately model the crystallin protein milieu as well as the intact lens itself. Since α-crystallin composes the majority of the lens soluble protein and is known to be involved in cataract pathogenesis, it is important to determine the range of interactions that involve α-crystallin under native conditions.

In this study, we used affinity-tagged human αA-crystallin, supplied as a transgenic protein to lens fiber cells, as bait to identify interacting proteins in the intact lens. To capture specific, weak, and/or transient interactions between α-crystallin and substrate proteins, we used formaldehyde as a cross-linking agent to produce stable protein complexes. Given the chemical stability of formal-dehyde-mediated cross-links, it was possible to purify α-crystallin complexes by affinity chromatography under denaturing conditions. This provided the added benefit of minimizing potential interactions with irrelevant proteins that may have transiently unfolded during tissue disruption and cell lysis.

Results from SEC analysis of transgenic lens homogenates demonstrated that 7×His-αA is integrated into the pool of α-crystallin oligomers. This indicates that proteins isolated by affinity chromatography after chemical cross-linking would derive from interactions involving the general population of α-crystallin complexes rather than a segregated pool of α-crystallin complexes dominated by the epitope-tagged subunits.

Identification of proteins associated with the α-crystallin complexes was achieved by mass spectrometry. This approach made it possible to identify binding partners through a global search rather than by probing for interactions using candidate binding partners as carried out previously (8, 31, 32). As expected, endogenous mouse αA-, αA^{ins}-, and αB-crystallin subunits were identified as major components of the affinity-purified α-crystallin complexes. This is consistent with our SEC analysis of transgenic lens homogenates, which demonstrated that 7×His-αA is integrated into the pool of native-sized α-crystallin oligomers. Both β- and γ-crystallins were also identified in these complexes. These results are consistent with previous in vitro (16) studies and a pattern of an increased level of aggregation of β- and γ-crystallins in lenses from α-crystallin knockout mice (7, 34, 35).

The cytoskeletal proteins actin and vimentin, which have previously been shown to interact with αA-crystallin from in vitro studies (8, 10), were identified in crystallin complexes. As these highly abundant lens proteins were also found in α-crystallin complexes purified from nontransgenic control lenses, further study will be required to determine if their co-isolation

reflects specific interactions with α-crystallin or other proteins associated with the complex after cross-linking.

Given the documented association of α-crystallin with membrane preparations (36, 37), we were initially surprised that membrane proteins were not identified in the affinity-purified complexes. The extent of association of α-crystallin with fiber cell membranes in human and bovine lenses is relatively low at early ages but appears to increase with age (13, 37, 38). For the study presented here, we used animals that were fewer than 6 weeks of age, so it is possible that the association of α-crystallin with membrane elements had not yet reached a sufficiently high level to be revealed in our cross-linking studies. The absence of membrane proteins among interacting proteins identified in the proteomic screen is consistent with our previous hypothesis that interactions between α-crystallin and lens membranes are not dependent on membrane proteins (15).

Affinity-isolated crystallin complexes from transgenic lenses, but not similarly treated nontransgenic control lenses, contained a unique protein identified as GRIFIN (galectin-related interfiber protein). Unambiguous identification was achieved using tandem mass spectra from peptides derived from two independent preparations of lens proteins. Identified previously by differential cDNA analysis of lens gene expression (21), GRIFIN is a 32 kDa homodimer of 16 kDa subunits. According to sequence homologies, GRIFIN is related to the galectin superfamily of proteins. However, unlike conventional galectins, GRIFIN lacks lactose binding activity due to sequence divergence at two positions (N48K and R72V) known to be required for β-galactoside binding (39). GRIFIN appears to be a cytoplasmic protein (data not shown), accounting for up to 0.5% of the total lens protein (21). In recent studies, GRIFIN was identified as a major protein in the insoluble fraction from rat nuclear fiber cells, presumably due to association with membrane elements (40). However, we prefer an alternate view in which interactions of GRIFIN with α-crystallin lead to formation of high-molecular mass complexes that become deposited over time among insoluble protein aggregates in the aging lens.

The MS/MS results were derived from cross-linked transgenic lenses. To control for the possibility that those two factors may have artificially induced an interaction between GRIFIN and α-crystallin, we examined a nontransgenic, non-cross-linked mouse lens homogenate. SEC can be used to separate the homogenate into the four major crystallin family members (Figure 1). Analyzing the peak fractions for GRIFIN by Western blot confirmed that GRIFIN eluted at a volume corresponding to the molecular mass of the dimer, but a portion co-eluted with α-crystallin at an apparent molecular mass of 600 kDa (Figure 4). A similar elution pattern was seen for γ-crystallin, a different protein known to interact with α-crystallin (16).

A membrane filtration assay was used to monitor the in vitro binding of α-crystallin to GRIFIN. Binding reaction mixtures including GRIFIN and α-crystallin were incubated at 37 °C until equilibrium was reached at 2 h. Previous experiments have shown that α-crystallin substrate binding is dependent on ATP concentration (16), so we first set out to determine whether ATP influenced interactions with GRIFIN. While holding recombinant α-crystallin and GRIFIN at a fixed concentration, binding reactions were set up in the presence of increasing amounts of ATP. In comparison to binding in the absence of ATP, we observed an approximately 5-fold increase in the level of binding of GRIFIN to αA-crystallin and the 3:1 αA:αB hetero-oligomer when ATP was included at a concentration of 3 mM. Stimulation of binding was also seen with αB-crystallin and bovine α_L-crystallin, albeit at an~2-fold increase following incubation with 3 mM ATP. These results are consistent with previously published studies showing enhancement of interactions of α-crystallin with γ-crystallin when measured in the presence of 3 mM ATP (16).

We note that maximal stimulation of GRIFIN binding by α-crystallin occurs at physiological concentrations of ATP. A gradient of ATP exists across the lens from ~1 mM in the core to ~4 mM in the cortex and ~6m Min the capsule-epithelium (41). This suggests that fluctuations in ATP levels may play a regulatory role inmodulating α-crystallin-GRIFIN interactions. While it has been shown that the binding of ATP induces exposure of hydrophobic sites and stabilizes the α-crystallin structure (16, 42, 43), additional studies will be required to elucidate the mechanism behind ATP-dependent enhancement of GRIFIN binding by α-crystallin.

To more fully characterize the binding interaction, we created a binding isotherm by incubating various amounts of GRIFIN with α_L-crystallin purified from calf lenses. The hyperbolic nature of the curve allowed nonlinear least-squares analysis using an independent and identical binding site model. The dissociation constant for this interaction is 6.5 ± 0.8 μM, which is similar to values seen previously for the binding of α-crystallin to other substrate proteins (16). It also re-confirms the lens homogenate SEC results where GRIFIN exhibited an elution profile similar to that of γ-crystallin, an α-crystallin interacting protein with similar binding affinity (16). The stoichiometry in terms of monomeric concentrations of crystallin is 0.25 ± 0.01. This indicates that one GRIFIN monomer binds a tetramer of α-crystallin subunits, consistent with a model that proposes a tetramer or smaller multimer as the binding competent form of the sHSPs during chaperone activity (44, 45). This equilibrium exchange between the large oligomer and the smaller multimer may be necessary for α-crystallin to bind a variety of substrate molecules, not just those that are unstable. We observed similar stoichiometric values when binding studies were carried out using oligomers containing only αA- or αB-crystallin. However, unlike the case for native α-crystallin, the binding data were not compatible with a model that assumed independent and identical binding (data not shown). The mechanism(s) behind this difference in binding will require further investigation.

Numerous studies have demonstrated that α-crystallin, like other sHSPs, has a chaperone-like ability to bind a wide variety of proteins if they become partially unfolded or thermodynamically destabilized (2). In contrast, our current studies show that destabilization of GRIFIN by heat or structural perturbation is not necessary for it to be bound with a relatively high affinity by α-crystallin. These results suggest that interactions between α-crystallin and GRIFIN may reflect a fundamentally different biological purpose than chaperone-like binding of α-crystallin to destabilized or partially denatured proteins.

Since GRIFIN comprises approximately 0.5% of the water-soluble lens protein (21), it is rather remarkable that so little is known about a possible physiological role for this protein in the lens. The high tissue concentration of GRIFIN suggests that it may contribute to the refractive index necessary for the lens to focus light on the retina. As previously suggested for conventional crystallins, we hypothesize that interactions between α-crystallin and GRIFIN may represent a mechanism for facilitating order and efficient packing of protein in the concentrated lens cytoplasm (46). Such interactions likely serve to maintain lens transparency and high refractive index.

While virtually nothing is known about a functional role for GRIFIN, previous studies have shown that other members of the galectin family are expressed in a spatially regulated pattern in the lens. Galectin-1 is synthesized in epithelial cells (47), whereas galectin-3 is found predominantly in differentiated fiber cells of the adult lens. The galectins are known to play important roles in mediating cellular interactions with extracellular matrix elements (48). In lens development, dynamic interactions between membrane-associated complexes of fiber cells and the underlying lens capsule appear to be crucial for proper cell elongation and organization of sutures (49, 50). Given that GRIFIN and α-crystallin have been shown

to associate with the plasma membrane of lens fiber cells (21, 37), it is possible that formation of complexes involving these proteins may influence cell elongation and suture formation during lens development. Indeed, lens growth and suture formation are markedly attenuated in α-crystallin deficient mice (34). Further studies are needed to establish whether α-crystallin is capable of binding to other members of the galectin family in vivo and whether interactions with the galectins help to facilitate the elongation and migration of fiber cells during lens development.

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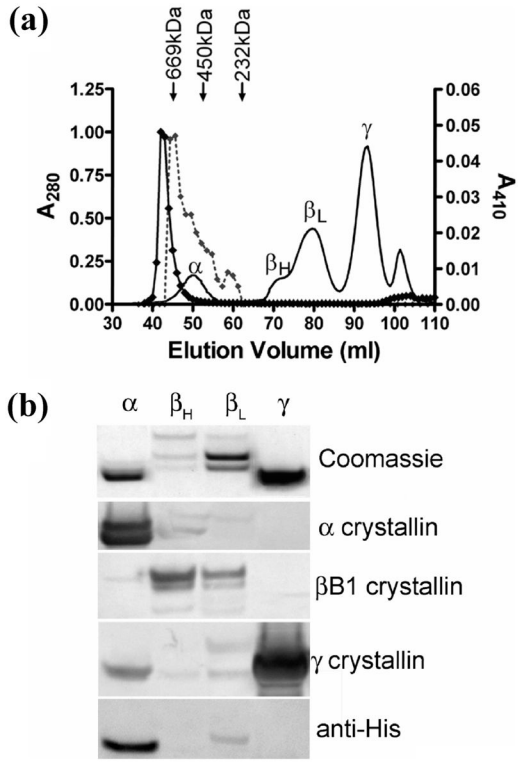

Figure 1.

His-tagged αA-crystallin subunits form complexes with endogenous proteins in the transgenic mouse lens. (a) Elution profiles following SEC of purified recombinant 7×His-αA (◆) and proteins extracted from the CRYAA^{WT} transgenic mouse lens (—) detected by in-line measurement of the absorbance at 280 nm (A_{280}). Elution of His-tagged αA subunits in the lens extract was monitored by an ELISA (A_{410}) on column fractions (◆ with a dashed line). Elution volumes for protein standards are indicated by arrows above the graph. Protein standards included thyroglobulin (669 kDa), ferritin (450 kDa), and catalase (232 kDa). (b) SDS–PAGE and Western analysis of pooled fractions corresponding to the elution volume of various crystallin family members as indicated on the profile in panel A (α, α-crystallin 43–65 mL; β_H, β_{high} crystallin 68–73 mL; β_L, β_{low} crystallin 73–86 mL; and γ, γ-crystallin 86–99 mL). The identity of each pool was confirmed by Western blotting using antibodies to α-crystallin, β_{B1}-crystallin, γ-crystallin, and the His-tag epitope.

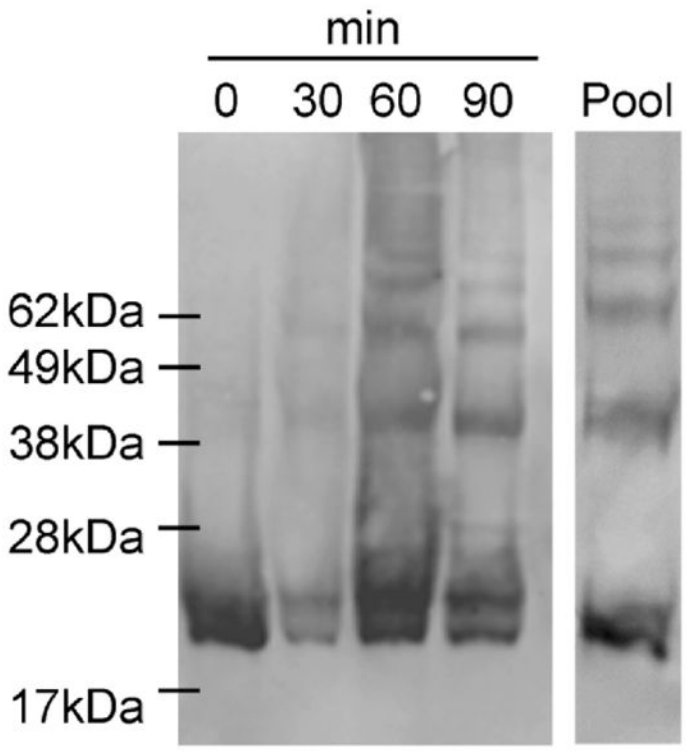

Figure 2.

Production and purification of cross-linked His-tagged α-crystallin complexes. Transgenic mouse lenses were incubated with 1% paraformaldehyde for the indicated period of time at 37 °C and homogenized in the presence of 6 M guanidine and ~1 M glycine. Homogenate samples from each time point were visualized by Western blots probed with the anti-His-tag antibody. Crude homogenates were loaded onto a His-Trap column to separate bulk lens protein from the His-tagged complexes. Fractions from the imidazole elution peak were pooled and analyzed for the presence of His-tagged protein ladder by Western blot.

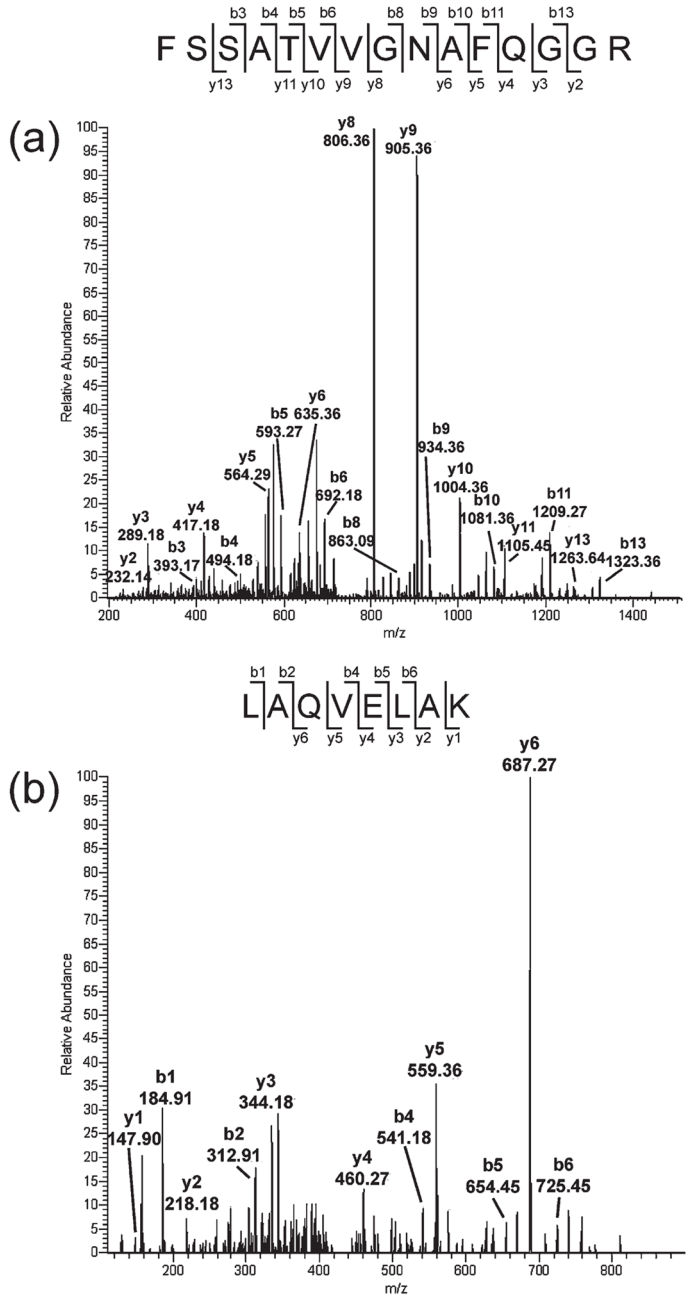

Figure 3.

Mass spectrometric peptide sequencing of GRIFIN. The tandem mass spectra corresponding to GRIFIN were recorded on the $[M+2H]^{2+}$ ions at m/z 749 (a) and m/z 436 (b). In the top section of each panel, the sequence of each peptide and the identified b (N-terminally derived fragment ions) and y (C-terminally derived fragment ions) ions are shown, corresponding to the masses indicated in the spectra. For both peptides, the sequences determined from the tandem mass spectra were assigned to GRIFIN through database searching.

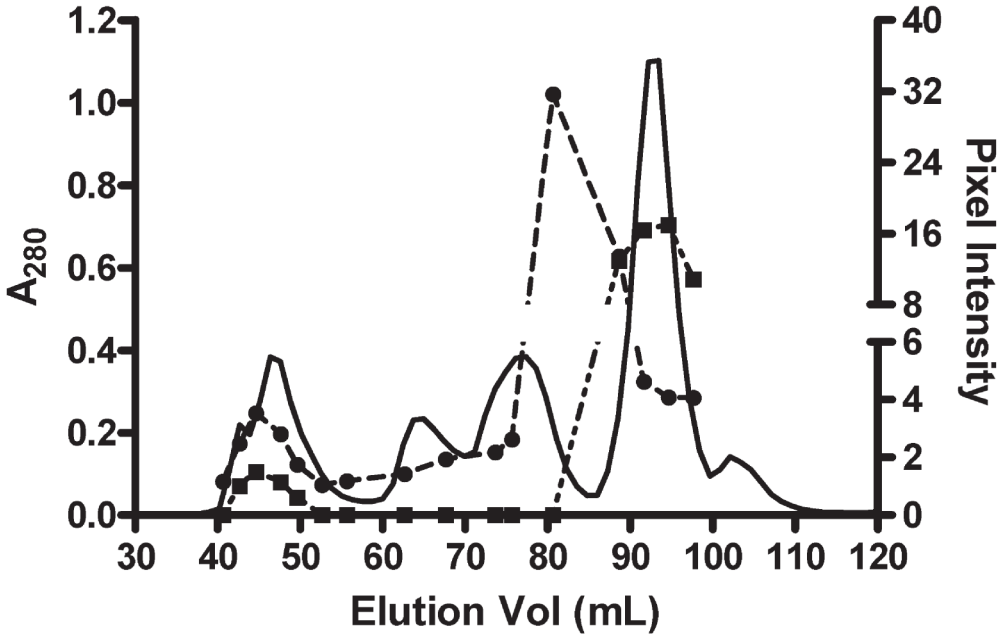

Figure 4.

Co-elution of substrate proteins with α-crystallin. The cleared supernatant from a wild-type mouse lens homogenate (not treated with cross-linker) was subjected to size exclusion chromatography. Elution fractions were measured for total protein [A_{280} (black trace)]. The abundances of γ-crystallin (■) and GRIFIN (●) were estimated by Western blotting of the indicated fractions. Blotting signals are plotted according to pixel intensity determined via ImageQuant.

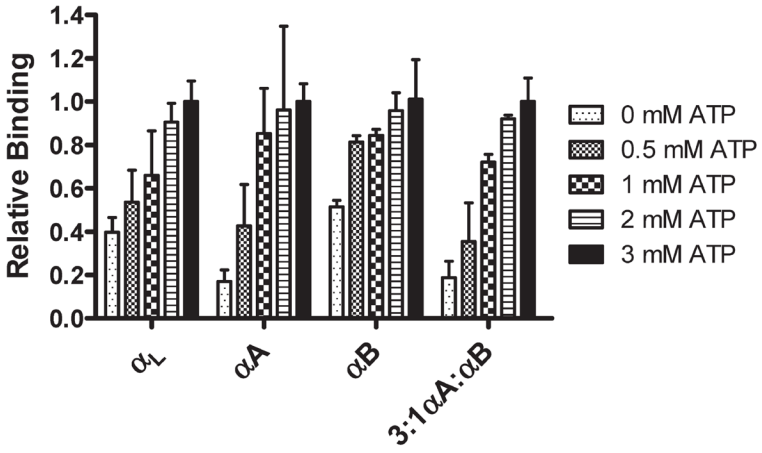

Figure 5.

Effect of ATP concentration on the binding of GRIFIN to α-crystallin. α-Crystallins (25 μM) were combined with 16 μM GRIFIN and ATP at the indicated concentrations. After incubation for 2 h at 37 °C, solutions were centrifuged through a 100 kDa molecular mass cutoff filter. Preliminary studies demonstrated that the α-crystallin subunits were completely retained by the membrane filter, allowing the amount of unbound GRIFIN to be determined by measuring the total protein in the filtrate using the BCA method. The amount of bound GRIFIN was calculated by subtracting the amount of unbound GRIFIN in the filtrate from the total amount of GRIFIN. Results for each time point were normalized to the amount bound at an ATP concentration of 3 mM. Crystallins came from the following sources. αL is native α-crystallin purified from bovine lenses. αA and αB are human recombinant αA- and αB-crystallins, respectively. 3:1 αA:αB represents complexes reconstituted from recombinant human αA and αB subunits in a 3:1 ratio.

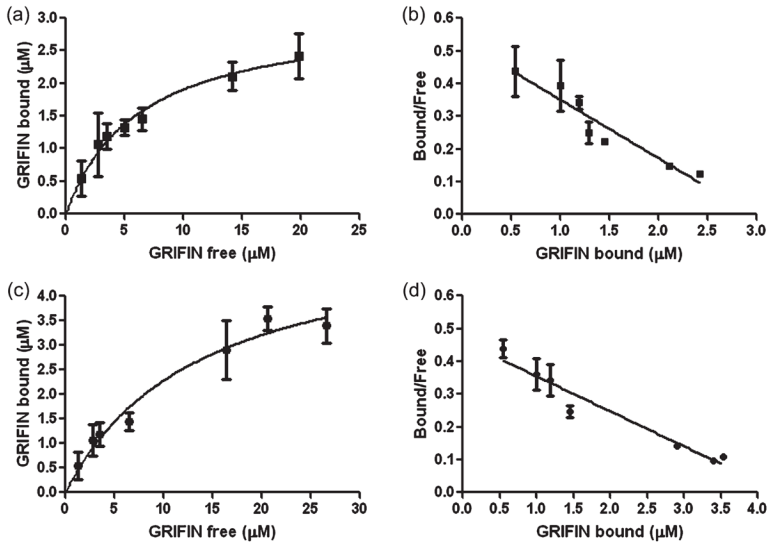

Figure 6.

Analysis of the binding interaction of GRIFIN with purified bovine α_L-crystallin (a and b) and purified mouse α_L-crystallin (c and d). Varying amounts of GRIFIN were incubated with 12.5 μM bovine or mouse α_L-crystallin at 37 °C for 2 h and processed as described in Materials and Methods. Binding isotherms (a and c) were created by plotting the amount of GRIFIN bound vs the amount of GRIFIN free. The solid line is the nonlinear least-squares fit to a model for identical and noninteracting binding sites. For bovine α_L-crystallin, the dissociation constant, K_d, and stoichiometry of binding calculated for the curve (a) were 6.52± 0.08 μM and 0.25 ± 0.01, respectively. The linear Scatchard analysis of this data set (b) yielded similar values with an r^2 value of 0.93. For mouse α_L-crystallin, the isotherm analysis (c) yielded a slightly higher K_d (13.6±5.3 μM) and a stoichiometry of 0.4 ± 0.08 GRIFIN molecule per α-crystallin monomer. Similar values were determined when the data were analyzed by Scatchard analysis (d) with an r^2 value of 0.86.

Table 1

Identities of Proteins Found To Be Associated with Purified His-Tagged αA-Crystallin Complexes by Liquid Chromatography and Tandem Mass Spectrometry

gi number	protein name	protein score[a]	no. of peptides
4503055	crystallin, αA (*Homo sapiens*)	403	23
33989574	crystallin, βA4 (*Mus musculus*)	331	16
6681035	crystallin, βB2 (*M. musculus*)	311	28
117351	α-crystallin A chain	289	16
73994848	predicted, similar to crystallin, βB3 isoform 5	244	16
229516	crystallin, αA	238	18
37589234	crystallin, βB3 (*M. musculus*)	237	10
6753530	crystallin, αB (*M. musculus*)	200	12
14285308	βA1-crystallin	171	11
76827509	crystallin, βA1 (*M. musculus*)	165	20
55408	vimentin (*M. musculus*)	149	4
929828	γ-crystallin D type	112	13
12963789	crystallin, βB1 (*M. musculus*)	110	5
33989588	crystallin, γB (*M. musculus*)	97	7
50262256	β-crystallin, unnamed (*M. musculus*)	94	9
31088965	crystallin, βA2 (*M. musculus*)	87	3
117459	γ-crystallin A (γ-crystallin 1-1)	86	8
92259	γ-crystallin 2-1-rat	85	8
33991687	crystallin, γF (*M. musculus*)	84	9
51507405	actin	82	3
33990600	crystallin, γC (*M. musculus*)	67	6
94376901	galectin-related interfiber protein (*M. musculus*)	66	2

[a]Protein scores are the sums of the unique ion scores for each peptide, which provide a numerical value to the probability of the sequence being unique to an individual protein.

Table 2

Identities of Proteins Found To Be Associated with His-Trap Purified Protein Complexes from Nontransgenic WT Control Cross-Linked Lens Homogenates by Liquid Chromatography and Tandem Mass Spectrometry

gi number	protein name	protein score[a]	no. of peptides
6681035	crystallin, βB2 (*M. musculus*)	106	3
28396657	actin	104	3
203474	creatine kinase	92	3
117336	α-crystallin A chain	79	5
133742	40S ribosomal protein S12	75	4
2338452	βA3-crystallin	68	2
12963789	crystallin, βB1 (*M. musculus*)	66	2
399412	elongation factor 1-α (EF-1-α)	62	2
10121625	60S ribosomal protein P1	60	3
73994846	predicted, similar to crystallin, βB3 isoform 4	57	2

[a]Protein scores are the sums of the unique ion scores for each peptide, which provide a numerical value to the probability of the sequence being unique to an individual protein.