Identification of Age- and Cataract-Related Changes in High-Density Lens Protein Aggregates

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Purpose. Cataract is believed to be caused by protein-protein and protein-membrane aggregation in the eye lens. After middle age, there is extensive binding of crystallins to the lens cell membranes as evidenced by sedimentation at high densities. Multiple protein modifications have been linked with cataract, whereas others have been associated with aging. The purpose of this study was to characterize protein constituents within high density protein-membrane fractions from normal aged or cataractous lenses and to compare these proteins and their modifications.

Methods. The inner nuclear regions of cataract or age-matched normal lenses were homogenized and proteins were separated using sucrose density gradient centrifugation. The low-density fractions (LDFs) and high-density fractions (HDFs) were analyzed by mass spectrometry using both top-down matrix-assisted desorption/ionization-mass spectrometry (MALDI-MS) and bottom-up liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based proteomic methods. Quantification of low molecular weight crystallin peptides, deamidation, and isomerization were performed.

Results. Compared with normal aged-lenses, membrane-associated protein aggregates in high density fractions of cataract lenses exhibited significantly higher levels of γ-crystallins, as well as γS- and γD-crystallin C-terminal peptides. Deamidation of γ-crystallin, but not of β-crystallin, was increased in cataract lens membrane-bound aggregates. A very high level of Asp isomerization was detected in bound α-crystallins from both aged and cataract lenses.

Conclusions. Binding of crystallin aggregates to human lens cell membranes is associated with protein truncation, deamidation, and isomerization, and was observed in normal aged and cataract lenses. However, the protein aggregates bound to membranes in cataract lenses exhibit distinct modifications to γ-crystallins that may arise as a consequence of additional protein degradation.

Keywords: gamma crystallin (γD-crystallin), gamma-S crystallin (γS-crystallin), cataract, sucrose density gradient centrifugation, crystallin peptides, deamidation, isomerization, truncation

Protein insolubility and protein aggregation are believed to be the basic pathogenic factors for a wide class of protein condensation diseases, including sickle cell anemia, diabetes, and many neurodegenerative diseases.[1,2] Cataract is also considered a protein condensation disease.[3] Protein condensation often leads to the formation of toxic protein aggregation and nucleation of amyloid fibril formation.[2] In the ocular lens, high molecular weight (HMW) protein aggregates can reach a molecular weight greater than 150×10^6 dalton (Da),[4] and, based on light scatter theory, protein aggregates with size 50×10^6 Da or greater can cause light scattering.[5] Under an electron microscope, protein packing in the lens cytoplasm and optical density appears homogenous in transparent lenses, but a marked density fluctuation in cataract lenses is observed.[6] Furthermore, denatured proteins can bind cell membranes and serve as nucleation sites for further aggregation.[7,8] Indeed, amyloid fibril formation of lens crystallins has been previously reported in vitro[9,10] and in mouse models of cataract.[11] These large protein-protein and protein-membrane aggregates cause local fluctuations in density and light scattering.[3] With time, increasing light scattering results in a continual increase in turbidity of the lens, loss of visual acuity, and, eventually, the development of age-related cataract.[4,12,13] Protein binding to the membrane is also thought to block membrane pores and create a barrier for transporting antioxidants to the center of the lens thereby disrupting lens homeostasis[14] and leading to massive oxidation of lens proteins: a characteristic feature of age-related nuclear cataract.[15]

Lens protein aggregation starts from early life and, strikingly, was found to increase in the lens nucleus after the age of 40 years.[4,14] Using sucrose density gradient centrifugation, multiple high-density bands, corresponding to membrane-bound protein aggregates, were only present

in samples from lenses over 40 years old and not in young lenses.[16] The mechanisms of protein aggregation have been extensively studied and multiple age-related modifications that are associated with protein aggregation have been identified including: deamidation,[17–21] truncation,[22] oxidation,[13,23] and non-native disulfide bond formation.[13,24] Such modifications are believed to destabilize the native structure and cause proteins to unfold.[19,25] In addition, there is a strong correlation between the accumulation of low molecular weight (LMW) crystallin fragments and protein aggregation.[21,22,26,27] The most studied crystallin fragment is αA-crystallin peptide 66-80 which was found to not only inhibit the chaperone activity of α-crystallin but also cause protein aggregation by forming a β-amyloid fibril-like structure.[26] In addition to LMW fragments, HMW complexes were found to have increased membrane-binding properties.[28]

The protein structures and mechanisms of aggregation are not completely understood. HMW aggregates initially are present in soluble fractions and these soluble HMW aggregates are believed to be precursors for insoluble aggregates formed with age.[13,29,30] However, it was also reported that the formation of these soluble HMW aggregates could be an important protective mechanism in the lens because soluble HMW aggregates are believed to be composed of large complexes of α-crystallin chaperones with other denatured lens proteins. The formation of these soluble HMW complexes could prevent uncontrolled aggregation and insolubilization.[31] Therefore, similar to the protein condensation process reported previously,[2] protein aggregation in the lens may involve the formation of functional condensed states and dysfunctional or toxic protein assemblies. This concept is supported by the fact that the majority of individuals can maintain transparent lenses for many decades of life, whereas only some individuals develop cataracts despite the ubiquitous occurrence of protein aggregation in lens with age. To understand cataractogenesis at a molecular level, it is crucial to identify the molecular components and their modifications within the protein aggregates formed in healthy and cataractous lenses. Differences in the aggregates formed in healthy and cataractous lenses have been reported[13,24] including differential mobility on agarose-polyacrylamide gels,[24] increased formation of non-native disulfide bonds,[13] and extensive oxidation of Met and Cys residues.[13] However, comprehensive characterization of proteins and protein modifications involved in protein aggregation in high density protein-membrane aggregates from healthy lenses and cataract lenses has not been done. In this study, we applied sucrose density gradient centrifugation to separate healthy and cataract lens samples into low-density fractions (LDFs) and high-density fractions (HDFs). Density-gradient centrifugation has been shown to effectively separate different forms of protein aggregates in brain[25] and has been used to show profound changes in lens protein-membrane aggregation with age.[16] Compared to commonly used fractionation based on solubility, sucrose-density gradient centrifugation provides more homogeneous fractions at different levels of aggregation. Thus, the presence of bands at different densities indicates a progressive process in protein-membrane aggregation. Through proteomic analysis of sucrose density fractions using mass spectrometry, we identified proteins that are associated with HDFs in both healthy and cataract lenses as well as those that were significantly elevated in HDFs in cataract lenses.

Methods

Sucrose Density Gradient Centrifugation

Healthy human lenses were acquired from the National Disease Research Interchange (NDRI; Philadelphia, PA, USA) and cataract lenses were obtained as a gift from Dr. Donita Garland (Ocular Genomics Institute at Massachusetts Eye and Ear, Harvard University, Cambridge, MA, USA). Four healthy lenses (73-year-old female patient; 71-year- old female patient; 66-year-old male patient; and a 63-year old male patient) and four cataract lenses (60-year-old male patient; 60-year-old male patient; 55-year-old female patient; and a 60-year-old female patient) were used. The human lenses were mounted on cryostat chucks and the anterior region (1 mm from the capsule) and the posterior region (1.5 mm from the capsule) were removed using a CM 3050 Cryostat (LEICA CM 3050S, Leica Microsystems Inc., Bannockburn, IL, USA) to yield about a 2-mm thick equatorial region. The inner nucleus region was obtained from the center of the section using a 4.5 mm diameter AcuPunch trephine (Acuderm Inc., Ft. Lauderdale, FL, USA). The rest of the lens tissue was saved at –80°C. The inner nucleus samples were homogenized in 10 mM Tris buffer containing 8% sucrose, 2 mM EDTA, and 2 mM DTT at pH 8.0, and further aspirated 30 times through a 25-gauge syringe needle. The sucrose density gradient centrifugation was performed as previously described[16] with slight modifications. Briefly, the sucrose density gradient was generated by sequential layering 1.5 mL of different sucrose concentrations (80%, 70%, 60%, 50%, 45%, and 25%) in 10 mM Tris buffer, 2 mM EDTA, and 2 mM DTT at pH 8.0 in Beckman thin wall centrifuge tubes, 14 × 89 mm. The sample, in 100 µL of 8% sucrose, was then carefully layered on the top of the gradient and an additional 2 mL of 8% sucrose solution was layered on the top of the sample. The samples were centrifuged in a SORVALL TH641 rotor at 100,000 g for 2 hours. Fractions were collected and the top 2 mL sample was defined as LDF (F0; Supplementary Fig. S1) and a 1.5 mL sample was collected from the interface between 70% and 80% sucrose as the HDF (F6; see Supplementary Fig. S1). Other fractions were collected centered from each interface, as shown in Supplementary Figure S1. Protein concentration was determined using the Coomassie Plus (Bradford) Protein Assay (Thermo Fisher Scientific, Rockford, IL, USA). Samples were saved at –80°C until further analysis.

Matrix-Assisted Desorption/Ionization-Mass Spectrometry Analysis

The HDFs (50 µL) were diluted 10 times with water and centrifuged at 100,000 g for 20 minutes. The pellets were suspended in 10 µL of 25% acetonitrile containing 0.1% trifluoroacetic acid (TFA). Samples (2 µL) were mixed with 2 µL of either 10 mg/mL 2,6-dihydroxyacetophenone (DHA) or saturated α-cyano-4-hydroxycinnamic acid (CHCA) matrix in 70% acetonitrile (0.1% TFA). The LDFs (2 µL) were directly mixed with 2 µL of the above matrix solution. Samples (1 µL) were spotted on the matrix-assisted desorption/ionization (MALDI) plate and analyzed using a Bruker Rapiflex Tissuetyper (Bruker Daltonics, Billerica, MA, USA) equipped with a Smartbeam 3D 10 kHz 355 nm Nd:YAG laser. A linear external calibration was applied to the instrument before data collection using protein standards (bovine insulin, equine cytochrome c, bovine ubiquitin I, and equine

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myoglobin; Bruker Daltonik). Mass spectrometric analyses were performed in the linear, positive mode with a mass range from 5000 to 25,000 Da. The LMW signals were also measured using a Bruker SolariX 15T FT-ICR mass spectrometer (Bruker Daltonics, Billerica, MA, USA) in positive ion mode with a Smartbeam II 2 kHz Nd:YAG (355 nm) laser. The mass spectrometer was externally calibrated prior to analysis using a peptide mixture (Leu-enkephalin, Angiotensin II, Fibrinopeptide B, ACTH fragment [1-24], and insulin chain B). The mass spectrometer was operated in positive ion mode and data were collected from m/z 700 to 5000 at a mass resolution of ~125,000 at m/z = 1046.542.

Sample Preparation for Liquid Chromatography-Tandem Mass Spectrometry Analysis

To extract LMW crystallin peptides from sucrose density fractions, 50 µg of total protein in LDF and HDF were precipitated with chloroform/methanol. After the pellets were dried, 5 µL of 10 mM NaOH, 10 µL of 25% acetonitrile, and 35 µL of 0.1% formic acid were sequentially added to the samples and the samples were vortexed after each addition. The samples were further diluted with 5% acetonitrile (0.1% formic acid) to 200 µL. 6.25 µg of the protein were cleaned with StageTips, as described by Rappsilber et al.,[31] and the peptides were eluted with 45% acetonitrile (0.1% formic acid). The samples were dried in a speedvac. The samples were reconstituted immediately before the analysis by sequentially adding 5 µL 20 mM ammonium hydroxide, 5 uL of 10% ACN (0.1% formic acid), and 15 uL of 0.1% formic acid with vortexing between each addition. Then, 1.25 µg of total protein was loaded onto a C18 column for liquid chromatography-tandem accurate mass spectrometry (LC-MS/MS) analysis.

To prepare samples for bottom-up proteomic analysis, 25 µg of total protein was reduced by adding dithiothreitol (DTT) to a concentration of 10 mM and incubated at 56°C for 45 minutes. Iodoacetamide (IAA) was then added to a final concentration of 55 mM and the samples were incubated at 25°C for 45 minutes. The proteins were precipitated with chloroform/methanol and digested by trypsin in 50 mM Tris buffer, pH 8.0 overnight. The samples were cleaned by StageTips and the peptides were eluted with 70% acetonitrile (0.1% formic acid). The samples were dried in a speedvac, and saved at –20°C in a freezer until analysis. The samples were reconstituted in 0.1% formic acid immediately before LC-MS/MS analysis.

LC-MS/MS Analysis

To quantify γS-crystallin fragments in HDFs and LDFs, the samples were separated on a one-dimensional fused silica capillary column (250 mm × 100 µm) packed with Phenomenex Jupiter C18 resin (3 µm mean particle size, 300 Å pore size) on an EASY-nLC 1000 system (Thermo Scientific, San Jose, CA, USA) with the following gradient at a flow rate of 0.4 µL/minute: 0 to 2 minutes: 2% ACN (0.1% formic acid), 2 to 37 minutes: 2% to 35% ACN (0.1% formic acid), 37 to 62 minutes: 35% to 95% ACN (0.1% formic acid) balanced with 0.1% formic acid in water. The eluate was directly infused into an LTQ Orbitrap XL mass spectrometer (Thermo Scientific, San Jose, CA, USA). The instrument was operated in a six-step data-dependent mode with one precursor scan event in Orbitrap (R = 30,000) to identify the top five most abundant ions for fragmentation in a linear ion trap. MS AGC target was set to 5E5 and MS2 AGC target was 1E4 and Dynamic exclusion (repeat count 2, exclusion duration 15 seconds) was enabled.

To analyze trypsin-digested samples, the samples were separated on a one-dimensional fused silica capillary column (250 mm × 100 µm) packed with Phenomenex Jupiter C18 resin (3 µm mean particle size, 300 Å pore size) coupled with an UltiMate 3000 RSLCnano system (Thermo Scientific, San Jose, CA, USA). A 78-minute gradient elution was performed, consisting of the following: 2 to 72 minutes, 2% to 40% ACN (0.1% formic acid); 75 to 78 minutes, 45% to 90% ACN (0.1% formic acid) balanced with 0.1% formic acid. The flow rate was 350 nL/minute. The eluate was directly infused into a Q Exactive Plus instrument (Thermo Scientific, San Jose, CA, USA) equipped with a nanoelectrospray ionization source. The data-dependent instrument method consisted of MS1 acquisition (R = 70,000) from m/z 350 to 1600, using an MS AGC target value of 3E6, maximum ion time of 80 ms followed by up to 20 MS/MS scans (R = 17,500) of the most abundant ions detected in the preceding MS scan. The MS2 AGC target value was set to 1E5 with a maximum ion time of 100 ms, HCD collision energy was set to 27, and dynamic exclusion was set to 10 seconds.

Data Analysis

For protein identification, the raw data from LC-MS/MS were loaded in MaxQuant software (http://maxquant.org/, version 1.6.6.0)[32] and searched against a Uniprot human reviewed database (downloaded on January 14, 2023). The following search parameters were used: enzyme: trypsin, maximum missed cleavage: 2, precursor mass tolerance: 5 ppm, variable modifications: protein N-terminal acetylation, Asn deamidation, and Met oxidation, maximum modification on a peptide: 2. A false discovery rate (FDR) of 1% was applied for both peptide and protein filtering. A Welch's *t*-test was performed to test statistical significance ($P < 0.05$).

Results

It is well known that the pattern of lens protein sedimentation is dramatically altered with human age.[16,33] Specifically, in sucrose gradient centrifugation, high density bands appear after middle age from lens nuclear samples. Molecular analysis of these bands revealed both lens crystallins and lipids suggesting that lens proteins become highly membrane bound with age. In the current study, sedimentation analysis, shown in Supplementary Figure S1, reproduced the previous findings where lens proteins sedimented to different opaque bands at different sucrose density interfaces (see Supplementary Fig. S1). Individual fractions were collected for proteomic analysis. In order to compare nuclear proteomes from aged and cataractous lenses, sucrose gradient fractions were analyzed by both matrix-assisted desorption/ionization-mass spectrometry (MALDI-MS) and by LC-MS/MS. The LC-MS/MS analysis was done without and with trypsin digestion to identify endogenous crystallin fragments and total protein content, respectively. After centrifugation, soluble proteins are found in the lowest fraction F0 and aggregated and membrane associated proteins are present in fractions (F1–F6). In this paper, only data from the LDF F0 and the HDF F6 are

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presented. Analysis of intermediate density fractions showed progressive changes (data not shown) and no systematic analysis was performed.

MALDI-MS Analysis

HDFs and LDFs from the inner nucleus region were analyzed using MALDI-MS over different m/z ranges to identify intact proteins at high mass (20–30 kilodalton [kDa]) and crystallin fragments at low mass (5–10 kDa). The major signals in LDFs match the molecular weights of intact human γ-crystallins with a singly charged ion at m/z 20876 and a doubly charged ion at m/z 10424. In addition, β-crystallins were also detected in LDF. However, due to possible post-translational modifications and limited mass resolution in this range, different forms of γ- or β-crystallins were not well-resolved (Fig. 1). No significantly different signals were detected in LDFs profiles between normal and cataract lenses, although a peak at m/z 10611—better resolved in the cataract sample—is likely a doubly charged truncated β-crystallin. The absence of intact α-crystallin signal in LDF is consistent with a previous MALDI imaging mass spectrometry report[34]; however, LC-MS/MS analysis confirmed the presence of α-crystallin in LDF. These results suggest that α-crystallins in LDF are present in HMW forms that are not easily detected by MALDI. Intact γ-crystallin signals were not detected in HDFs, instead αA-crystallin N-terminal truncation products were the major protein products detected (see Fig. 1). Results from measurements in the LMW range are shown in Figure 2 which show LMW crystallin fragments were present in HDFs of both cataract and healthy lenses. Major signals in HDFs of healthy lenses correspond to αA-crystallin peptides, αB-crystallin N-terminal peptides, and βA3-crystallin C-terminal peptides. In addition to these peptides, HDFs of cataract lenses showed strong signals from a γD-crystallin peptide 161-172 (ARVGSLRRVIDF) and a series of γS-crystallin C-terminal peptides. The 2 strongest signals correspond to γS-crystallin 144-177 (NYRGRQYLLDKKEYRKPIDWGAAS-PAVQSFRRIVE) and γS-crystallin 153-177 (KKEYRKPIDW-GAASPAVQSFRRIVE). These γS-crystallin peptides in HDFs from healthy lenses were very weak or barely detected. In fact, all LMW peptides were barely detected in LDFs from healthy and cataract lenses (data not shown). These results are consistent with previously reported results that γS-crystallin C-terminal peptides were exclusively detected in the cataractous region by MALDI imaging mass spectrometry.[35]

Relative Quantification of γS- and βA3-Crystallin C-Terminal Peptides in HDFs and LDFs of Normal and Cataract Lenses

MALDI-MS results showed strong signals of γS-crystallin C-terminal peptides in cataract lenses and strong signals of βA3-crystallin C-terminal peptides in both healthy and cataract lenses. Quantification of these crystallin C-terminal peptides in HDFs and LDFs was then performed using more sensitive LC-MS/MS analysis.

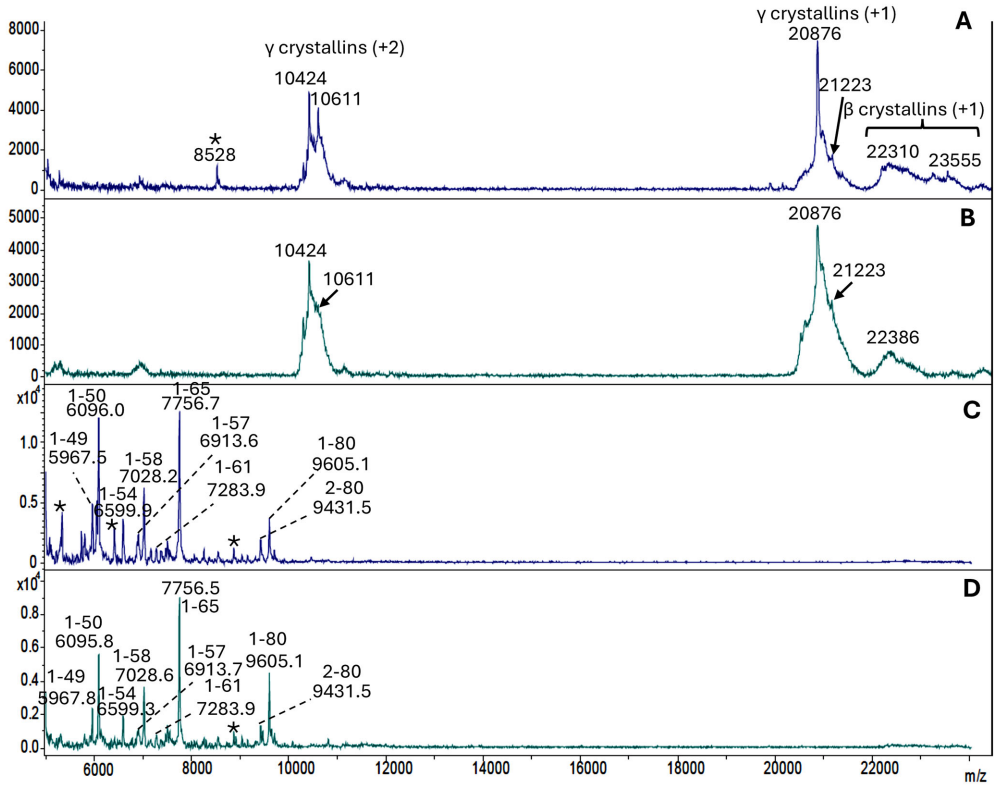

Figure 1. Representative MALDI-MS analysis of intact proteins and peptides in LDF (A, B) and HDF (C, D). HDFs and LDFs from normal lenses (**B, D**) and cataract lenses (**A, C**) were mixed with DHA matrix and analyzed by MALDI-MS using a Bruker Rapiflex Tissuetyper in linear positive mode (m/z range 5000–24,000). Representative spectra are shown from a 73-year-old healthy lens and 60-year-old cataract lens. The major signals in LDF corresponded to γ-crystallins (+1 or +2 charged) and β crystallins and the major signals in HDFs corresponded to truncated αA-crystallin fragments. * Indicates signals that have not been identified.

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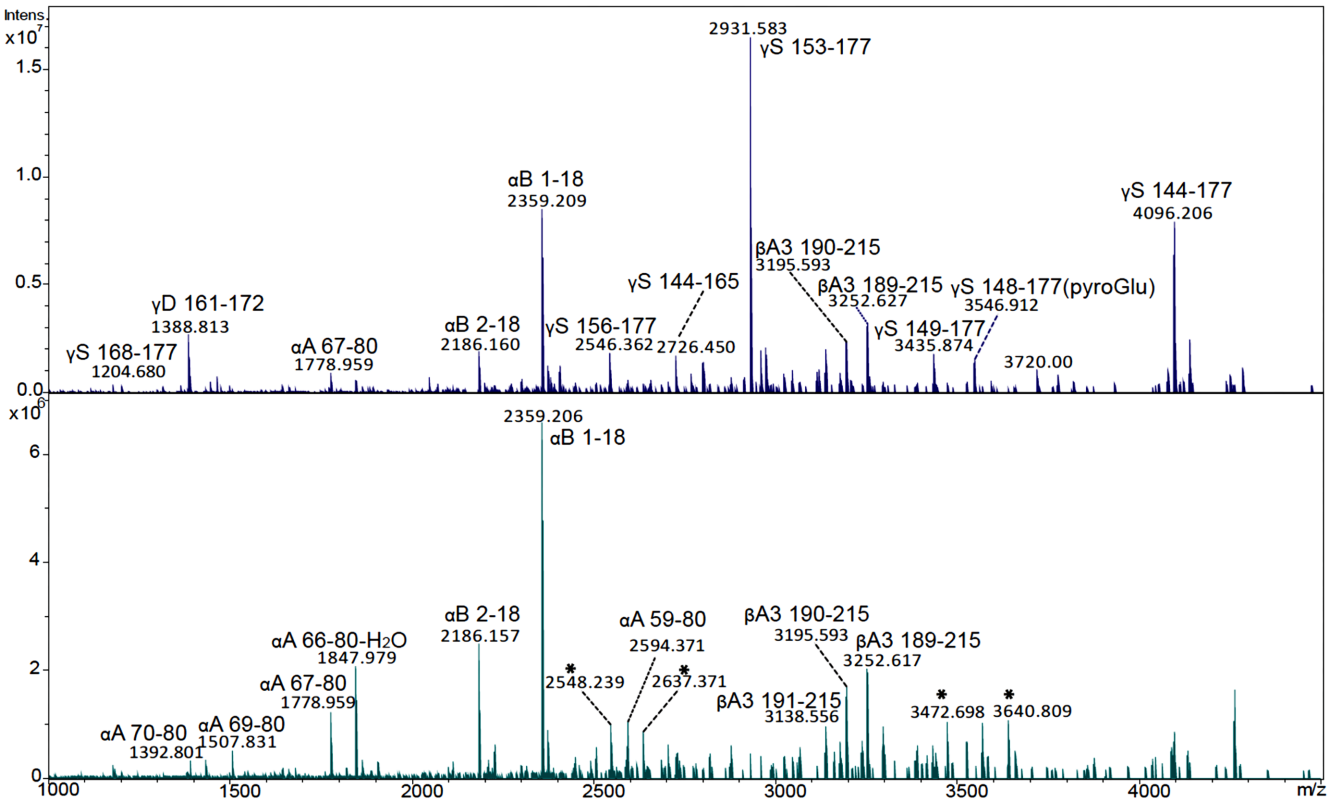

Figure 2. MALDI-MS analysis of HDF in low m/z range. HDFs from a healthy lens (73-year-old, *bottom*) and a cataract lens (60-year-old, *top*) were mixed with CHCA matrix and analyzed in a Bruker SolariX 15T FT-ICR mass spectrometer (m/z range 1000–5000). Strong signals from γS-crystallin 153-177 and γS-crystallin 144-177 and a series of other γS-crystallin peptides were detected in the HDF from cataract lenses whereas these signals were barely detected in HDF from healthy lenses. A series of βA3- and αA-crystallin peptides was detected in both normal and cataract lenses. * Indicates signals that have not been identified.

Consistent with MALDI-MS analysis, strong signals corresponding to γS-crystallin 144-177 and 153-177 were detected in HDFs of cataract lenses (Fig. 3A). In addition to these two peptides, a series of γS-crystallin C-terminal peptides (145-177, 146-177, 147-177, 148-177, 149-177, 150-177, 152-177, 154-177, 155-177, 156-177, 157-177, 162-177, 165-177, 166-177, 167-177, 168-177, and 169-177) were also detected in cataract lenses. These signals were either very weak or not detected in LDFs of healthy and cataract lenses.

With the higher dynamic range provided by LC-MS/MS analysis, signals corresponding to γS-crystallin 144-177 and 153-177 peptides can be detected in HDFs of healthy lenses, but the signals were significantly weaker than the signals in HDFs of cataract lenses. Based on the average results from 4 biological replicates in this study, signals of γS-crystallin 144-177 and γS 153-177 are 133- and 58-fold, respectively, higher in intensity in HDFs from cataract lenses compared to healthy lenses. Note that the data are plotted on a log2 scale indicating that signal intensity differences between samples are large. Quantification of the six major γS-crystallin peptides in HDFs and LDFs can be found in Figure 3A. Signals from all six peptides were statistically significantly higher in HDFs of cataract lenses compared with LDFs of cataract lenses or HDFs of healthy lenses. In healthy lenses, γS-crystallin 144-177 and 153-177 signals, although weaker than in cataract lenses, were also found to be significantly higher in HDFs than in LDFs. Signals of other γS-crystallin peptides in healthy lenses were very low and a trend of increasing abundance in HDFs was detected but the difference did not reach statistical significance. As a comparison, βA3-crystallin peptides were also quantified (Fig. 3B). In contrast to γS-crystallin C-terminal peptides, βA3-crystallin peptides can be detected in both the HDFs and LDFs of healthy and cataract lenses. Increased abundance of βA3-crystallin C-terminal peptides in HDFs can be detected in both healthy and cataract lenses compared with corresponding LDFs, and some of these peptides were also higher in the cataract lenses, however, the signals in HDFs from cataract lenses were normally less than four-fold higher compared with healthy lenses.

Protein Deamidation and Isomerization

To understand whether post-translational modifications were enriched in high density protein fractions, we compared protein modifications in HDFs with LDFs. Significant differences in the levels of deamidation were observed and the differences were large for many deamidation sites. To quantify the levels of deamidation for some major deamidation sites, the intensities of the deamidated peptide signals were divided by the non-deamidated peptide signals. The results are shown in Figure 4.

For the majority of deamidation sites, the levels of deamidation were significantly higher in the HDFs compared with the corresponding LDFs. Furthermore, deamidation on some sites was found to be significantly higher in HDFs of cataract lenses compared with HDFs of healthy lenses and these sites were all found on γ-crystallins. Even though

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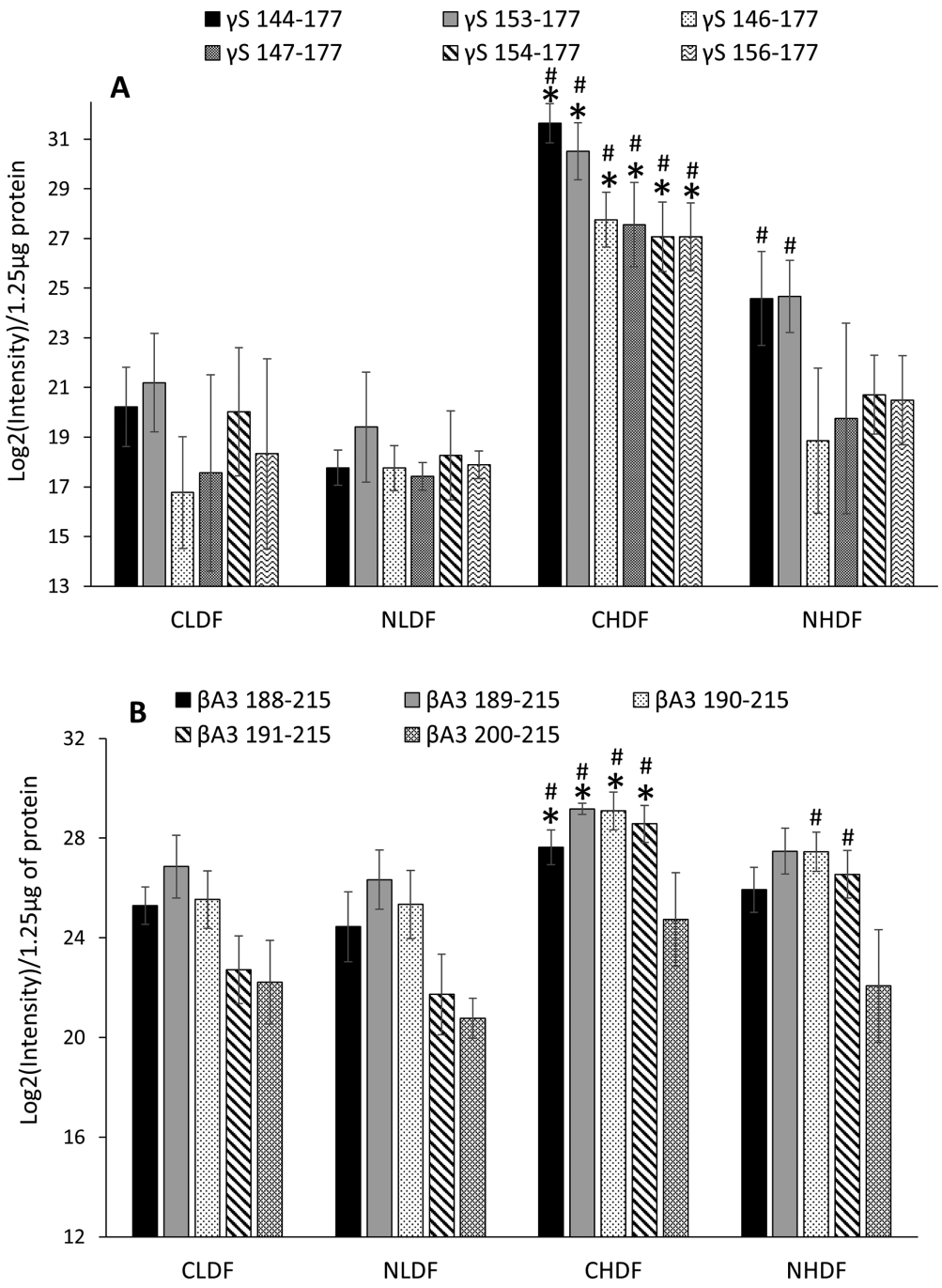

Figure 3. Quantification of γS- and βA3-crystallin C-terminal peptides in HDFs and LDFs. LMW peptides were extracted from HDFs and LDFs from both normal and cataract lenses. The γS-crystallin peptides (**A**) and βA3-crystallin peptides (**B**) were measured by LC-MS/MS. CLDF, cataract low density fraction; NLDF, normal low-density fraction; CHDF, cataract high density fraction; NHDF, normal high density fraction. The same abbreviations were used for other figures. Peaks corresponding to the peptides were extracted and peak intensities were used for quantification. # Indicates significantly different in HDFs than corresponding LDFs; * indicates significantly different in cataract lenses compared to corresponding fraction of normal lenses ($P < 0.05$). Data are presented as mean ± SD ($n = 4$).

many Asn residues on β-crystallins undergo high levels of deamidation, the levels of deamidation were not significantly different in healthy lenses compared with cataract lenses. In addition to Asn residues on β-crystallins shown in Figure 4, a similar trend was found for deamidation on βA3-crystallin N40, βA4-crystallin Q63, βB1-crystallin N68, and βB1-crystallin N 158. Deamidation on some sites (e.g. βB2-crystallin N116) was lower in cataract lenses than in healthy lenses. Deamidation at Gln residues in β-crystallins followed a similar trend to Asn deamidation, for example, in βA4-crystallin Q42 and βB1-crystallin Q70, deamidation was higher in HDFs compared to LDFs. Deamidation on βB1-crystallin Q70 was also significantly decreased in HDFs of cataract lenses compared with HDFs in healthy lenses (data not shown). There is no significant difference in the levels of deamidation between the LDFs of

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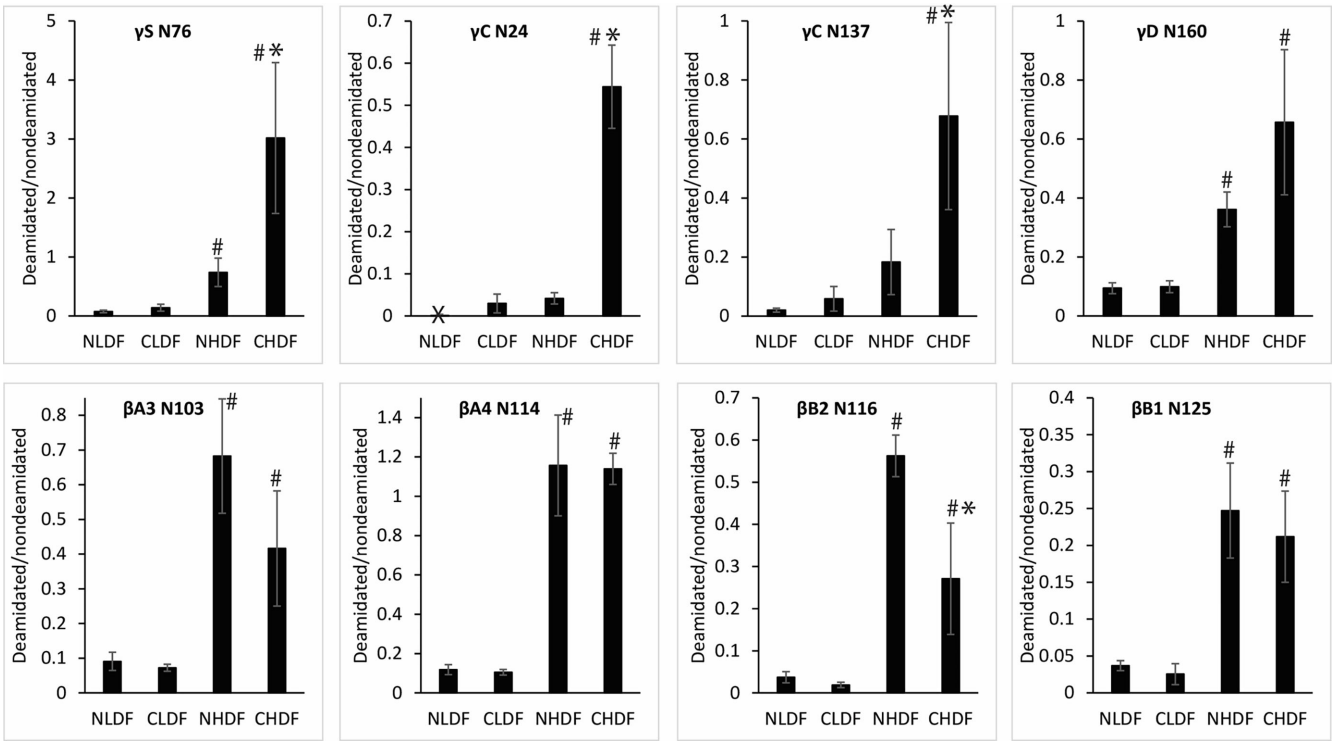

Figure 4. Quantification of Asn deamidation. The levels of deamidation were calculated as ratios of the intensities of deamidated peptides versus the intensities of their corresponding non-deamidated homologues. * Indicates significant difference between HDFs in cataract lenses compared with HDFs in healthy lenses; # Indicates significantly higher in HDFs compared with corresponding LDFs ($P < 0.05$). Data are presented as mean ± SD ($n = 4$).

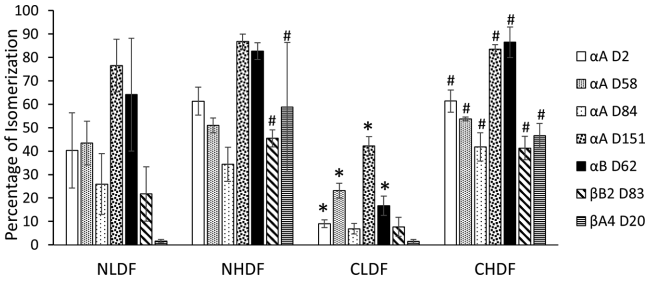

Figure 5. Percentage of Asp isomerization/racemization in alpha and beta crystallins from aged lenses. The total isomer peak intensity (I_{isomer}) was calculated by combing peak intensities of all isomers except L-Asp form and the total peak intensity was the summed peak areas of all peaks (I_{total}). The percent of isomerization was calculated by 100 × (I_{isomer}/I_{total}). # Indicates significantly higher in HDFs compared with corresponding LDFs; * indicates significantly lower HDFs in cataract lenses compared with the same fraction from healthy lenses ($P < 0.05$). Data are presented as mean ± SD ($n = 4$).

healthy lenses and cataract lenses for all deamidation sites analyzed.

Other prevalent modifications detected in these samples are Asp isomerization and racemization. It is well-known that extensive isomerization and racemization can occur on multiple Asp residues on αA- and αB-crystallin[36–39] and remarkably high levels of isomerization on these residues was also detected in this study (Fig. 5). Selected ion chromatograms (SICs) demonstrating extensive isomerization of the Asp 2 residue on αA-crystallin in HDFs and LDFs are shown in Supplementary Figure S2. Despite high levels of isomerization, no significant differences were observed in HDFs of cataract lenses. However, a surprisingly lower level of isomerization was found in LDFs of cataract lenses. Isomerization on some β-crystallin Asp residues (βB2 D83 and βA4 D20) was significantly higher in HDFs compared with LDFs in both healthy and cataract lenses, but no difference was detected between healthy and cataract lenses.

Discussion

Age is the primary risk factor for nuclear cataract and many changes previously identified in cataract lenses are also present in aged transparent lenses, such as protein aggregation,[4,40] deamidation and isomerization,[17–20] and oxidation.[14,41] The results from this study and a related study[35] suggest that modifications, distinct from age-related modifications, occur in cataract lenses. Moreover, our results suggest that protein aggregation and membrane binding in healthy aged lenses and cataract lenses could involve different proteins and different processes. Specifically, our results indicate that modifications to γ-crystallins could play a crucial role in cataractogenesis.

Comparative analysis of the HDFs in healthy and cataract lenses revealed significantly different protein and peptide signals in cataract lenses which include a series of γS-crystallin C-terminal peptides and a C-terminal peptide from γD-crystallin. Remarkably, γS-crystallin C-terminal peptides were 58- and 133-fold higher in cataract lens HDFs compared with HDFs from healthy lenses. This result is consistent with a previous report that γS-crystallin and γD-crystallin C-terminal peptides were only detected in cataract

lenses but not in healthy lenses by MALDI imaging.[35] With a better dynamic range in LC-MS/MS analysis and sucrose density gradient enrichment of HDFs, signals of γS-crystallin C-terminal peptides can be detected in HDFs of healthy lenses, but at significantly lower abundances than in cataract lenses. Additionally, results from the current study showed that these γ-crystallin C-terminal peptides were highly abundant in HDFs but barely detected in LDFs of cataract lenses, suggesting a pro-aggregatory property similar to the previously reported αA-crystallin peptide 66-80 (SDRDKFVIFLDVKHF).[26]

Like αA-crystallin 66-80, γS-crystallin C-terminal peptides are rich in basic and aromatic residues. Considering that peptides with basic and aromatic residues could bind with the membrane bilayer,[17] it is likely these peptides are strongly associated with the cell membrane. During sample preparation, we noticed that extraction of these peptides from the lens samples required either a basic solution or a higher concentration of acid in the buffer compared with standard proteomic sample preparation methods. Previously, it was reported that γD-crystallin can polymerize through successive domain swapping at C-terminal β-strands leading to age-onset cataract.[36] Therefore, it is reasonable to predict the membrane-associated γS-crystallin peptides could recruit γS-crystallin protein to the membrane and initiate a similar aggregation process as reported for γD-crystallin.[36] Consistent with this hypothesis, our proteomic results revealed an increase in the total intensities of γ-crystallin in HDFs, suggesting that, apart from the C-terminal peptides, the intact γ-crystallins were also more abundant in the membrane bound high-density aggregates.

β- and γ-crystallins, members of a βγ-superfamily, both consist of four Greek key motifs that fold to two compact domains; however, γ-crystallins are monomeric whereas β-crystallins form high-order multimers.[37] Results from this study support the notion that modifications on β-crystallins may induce age-related changes to the proteins, but that these changes were not significantly different between healthy and cataract lenses.

Considering the sequence homology between βA3-crystallin and γS-crystallins, and the fact that both proteins generate highly abundant C-terminal peptides in the lens, it is interesting that only the γS-crystallin C-terminal peptides were significantly higher in cataract lenses. The formation of C-terminal peptides may be due to the well-known non-enzymatic cleavage mechanism involving a succinimide intermediate.[42,43] The initial truncation of γS-crystallin can occur at residues N143 and D152 in γS-crystallin, as well as at D186 in βA3-crystallin. Subsequently, N-terminal amino acids are sequentially removed from these fragments, generating a series of C-terminal peptides, perhaps by aminopeptidase activity. Although the local environment in the native, folded γS-crystallin structure may prevent truncation at residues N143 and D152, unfolded γS-crystallin, which could occur in cataract lenses, may resemble the structure of βA3-crystallins and be susceptible to cleavage at these sites. Furthermore, the pattern of deamidation on sites of β-crystallin and γ-crystallins is also interesting. Increased deamidation on γS-crystallin Asn76 in cataract lenses is consistent with a previous report.[38] Importantly, Asn76 was reported as one of the most impactful residues for γS-crystallin aggregation by deamidation.[44] Previously, deamidation in γS-crystallin was found to be influenced by surface exposure[45] and increased deamidation on Asn76, a residue with low surface accessibility,[44] in cataract lenses may indicate that protein conformation changes occur preceding deamidation. The consequences of γS-crystallin deamidation were reported to include enhanced attractive protein–protein interactions due to introduction of additional negative charges,[46] and accelerated oxidation and disulfide bond formation.[21,47] Therefore, once deamidation occurs, it could lead to further protein aggregation and light scattering.[21,46,47]

In addition to deamidation, very high levels, up to 90%, of isomerization/racemization of Asp residues were detected in both aged healthy lenses and cataract lenses, which is consistent with a previous report.[48] However, the high level of isomerization was also found in the LDF of the healthy lens. The high levels of isomerization/racemization in the unaggregated proteins of the LDF of aged healthy lenses suggest that isomerization/racemization is an age-related process and may not contribute to widespread protein aggregation or membrane binding. Our results are in remarkable agreement with a previous report,[39] despite differences in sample preparation procedures (whole lens homogenates versus lens nuclear fractions). Further, we detected no differences in isomerization/racemization levels between proteins from healthy and cataract lenses found in HDFs. Our results for αA-crystallin Asp 58 differ from a previous report where increased D-isoAsp was observed for this residue in cataract lenses compared with transparent lenses.[38] Again, differences in sample preparation and fractions examined could contribute to the differences observed between studies, as well as the fact that we report total isomerization/racemization levels. Notably, both isomerization/racemization sites on β-crystallins (D83 of βB2-crystallin and D20 of βA4-crystallin) increased in the high-density fractions of healthy and cataractous lenses suggesting increased aggregation or membrane binding. Deamidation as well as isomerization of β-crystallins is predicted to lead to protein aggregation.[19,49] Although isomerization of βB2-crystallin D83 has been reported in the lens soluble protein fraction,[50] there was no effect of lens age on isomerization levels. Last, we observed lower levels of Asp isomerization/racemization in the LDF of cataract lenses compared with healthy lenses. The reason for this is unclear; however, we speculate that other modifications in cataract lenses may shift the isomerized proteins of the low-density fraction to other fractions, but not to the most aggregated state in high-density fractions.

In summary, major modifications to human lens γ-crystallins, including truncation and deamidation, are associated with lens protein aggregation and membrane binding in cataract lenses. These processes appear to be distinctly cataractogenic in that such modifications are present at very low abundances in age-matched healthy lenses.

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