Oligomerization with wt α A- and α B-Crystallins Reduces Proteasome-Mediated Degradation of C-Terminally Truncated α A-Crystallin

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PURPOSE. We previously demonstrated that the ubiquitin-proteasome pathway (UPP) is a general protein quality control system that selectively degrades damaged or abnormal lens proteins, including C-terminally truncated α A-crystallin. The objective of this work was to determine the effects of wt α A- and α B-crystallins on the degradation of C-terminally truncated α A-crystallin (α A₁₋₁₆₂) and vice versa.

METHODS. Recombinant wt α A, α B, and α A₁₋₁₆₂ were expressed in *Escherichia coli* and purified to homogeneity by chromatography. Subunit exchange and oligomerization were detected by fluorescence resonance energy transfer (FRET), multiangle-light scattering and coprecipitation assays. Protein substrates were labeled with ¹²⁵I and lens epithelial cell lysates were used as the source of the UPP for degradation assays.

RESULTS. FRET, multiangle light scattering, and coprecipitation assays showed that α A₁₋₁₆₂ exchanged subunits with wt α A- or wt α B-crystallin to form hetero-oligomers. α A₁₋₁₆₂ was more susceptible than wt α A-crystallin to degradation by the UPP. When mixed with wt α A-crystallin at 1:1 or 1:4 (α A₁₋₁₆₂ : wt) ratios to form hetero-oligomers, the degradation of α A₁₋₁₆₂ was significantly decreased. Conversely, formation of hetero-oligomers with α A₁₋₁₆₂ enhanced the degradation of wt α A-crystallin. The presence of α A₁₋₁₆₂, but not wt α A-crystallin, decreased the degradation of wt α B-crystallin.

CONCLUSIONS. α A₁₋₁₆₂ forms hetero-oligomers with wt α A- and α B-crystallins. Oligomerization with wt α A- or α B-crystallins reduces the susceptibility of α A₁₋₁₆₂ to degradation by the UPP. In addition, the presence of α A₁₋₁₆₂ in the hetero-oligomers also affects the degradation of wt α A- and α B-crystallins. (*Invest Ophthalmol Vis Sci.* 2012;53:2541-2550) DOI: 10.1167/iops.11-9147

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Lens fiber cells contain high concentrations of crystallins in the cytoplasm. These crystallins provide the lens with a high refractive index and minimize light scattering at the membrane-cytoplasm interface. The proper packing of these proteins in cells is essential for maintaining lens transparency.^{1,2} Disruption of the proper arrangement of these crystallins by adverse modifications is causally related to cataractogenesis.³⁻⁶ With aging and environmental insults, lens proteins undergo various modifications, such as oxidation, glycation, deamidation, and truncations.⁷⁻¹⁶ These modified or damaged proteins are usually unstable in aqueous solution and tend to aggregate and precipitate. The aggregation and precipitation of damaged/modified proteins accounts for a large portion of the age- and cataract-related increases in water-insoluble proteins in the lens.¹⁷⁻²⁴ Thus, efficient removal or repair of the damaged or adversely modified proteins before their aggregation and precipitation appears to be essential for lens transparency.

The ubiquitin-proteasome pathway (UPP) is an important protein quality control mechanism that selectively recognizes and degrades proteins with abnormal structures.²⁵⁻³¹ We and others have demonstrated that the lens has a fully functional UPP and that this proteolytic pathway selectively degrades various forms of damaged proteins, including oxidized, glutathiolated, and thermally denatured proteins,²⁵⁻⁴³ but not glycated proteins.⁴⁴ To establish and maintain lens protein homeostasis, the proper function of the UPP is essential for timely degradation of damaged, adversely modified proteins and many regulatory proteins. Disruption of the normal function of the UPP in the lens results in abnormal lens development and cataracts.⁴⁵

Truncation is one of the dominant forms of posttranslational modifications of lens proteins on aging and cataractogenesis.⁴⁶⁻⁵³ Although other proteases, such as β A3-crystallin or β A3-crystallin-associated proteinases may be involved in cleavage of lens protein,^{54,55} calpains play a major role in the generation of various truncated forms of crystallins.⁶⁰⁻⁶³ Calpains are a superfamily of structurally related, calcium-activated cysteine proteases.⁵⁶⁻⁵⁸ Calpain-mediated cleavage of lens proteins plays an important role in the aggregation and insolubilization of lens proteins, including crystallins and cytoskeleton proteins.^{24,59-64} Calpain-mediated cleavage also causes the loss of chaperone activity of α -crystallins.⁶⁵

In addition to calpain- or other protease-mediated degradation, lens proteins in long-lived species such as humans can also be truncated nonenzymatically. For example, a recent publication postulated that a major cleavage site in lens AQP0 was caused nonenzymatically.⁶⁶

Both α - and β -crystallins, but not γ -crystallins, are susceptible to calpain-mediated cleavage.⁶⁷ Whereas α -crystallins are cleaved by calpains at their C-terminus,⁶⁸ β -crystallins are cleaved closer to their N-terminus.⁶⁹ C-terminal cleavage of α A-crystallin by calpains can occur at several sites. The major cleaved products of

α A-crystallin in the rat lenses include αA_{1-151} , αA_{1-156} , αA_{1-163} , and αA_{1-168} .⁶⁸ αA_{1-162} can be generated by incubation of α A-crystallin with calpain-2 in vitro,⁶⁸ but it is barely detectable in normal rat lenses.^{68,70} However, αA_{1-162} is readily detected in diabetic cataract rat lenses and is also found in small quantities in the older fibers of normal human lenses.^{70,71} Although there is considerable evidence of calpain-mediated crystallin truncation in mouse or rat lenses, there is far less evidence for calpain-mediated cleavage in human lenses, because there are no active calpain-3 isoforms in human lenses.⁷² Calpain-2, the enzyme that may produce αA_{1-162} , has much lower activity in the human lens. Thus, the levels of αA_{1-162} in human lens are very low.⁷¹

Accumulation of truncated α A-crystallins in cataractous lenses can result from both an increase in production and/or a decrease in degradation of the truncated products by other proteases, such as the UPP. In a previous study we compared the susceptibility of wt and the C-terminally truncated α A-crystallins to UPP-mediated degradation.⁷³ We found that the sites of truncation at the C-terminus of α A-crystallin affect its conformation and susceptibility to UPP-mediated degradation. Whereas αA_{1-168} is degraded at a reduced rate as compared with wild-type (wt) α A-crystallin, αA_{1-162} is degraded at a much faster rate than wt α A-crystallin. Because the αA_{1-162} is less thermally stable and prone to aggregation, it would appear that the timely degradation of the αA_{1-162} is critical to prevent its accumulation and aggregation in the lens. The rapid degradation of αA_{1-162} by the UPP may explain why αA_{1-162} is barely detectable in normal lenses.

α -Crystallin normally exists as large heterogeneous oligomers composed of two types of subunits, α A-crystallin and α B-crystallin, in a molar ratio of 3 to 1 in most mammalian lenses.⁷⁴ It has been reported that C-terminally truncated α A-crystallins also form oligomers with wt α A- and α B-crystallins.⁷⁵ Our previous work demonstrated that isolated αA_{1-162} is rapidly degraded by the UPP,⁷³ but it remained unknown whether the αA_{1-162} in complexes with wt α A- or α B-crystallins was also selectively degraded. To fill this gap of knowledge, we investigated the effects of oligomerization with wt α A- or α B-crystallins on degradation of αA_{1-162} . Conversely, we determined the effects of incorporation of the αA_{1-162} into hetero-complexes on degradation of wt α A- and α B-crystallins. The data indicate that whereas oligomerization with wt α A- or α B-crystallin reduced the degradation of αA_{1-162} , incorporation of αA_{1-162} into wt α A-crystallin complex enhanced the degradation of wt α A-crystallin. In contrast, incorporation of αA_{1-162} into wt α B-crystallin complex reduced the degradation of wt α B-crystallin.

MATERIALS AND METHODS

Construction of C-Terminally Truncated α A-Crystallin

To mimic C-terminal cleavage by calpains, αA_{1-162} -crystallin was generated by PCR-based cloning.⁷³ Human α A-crystallin cDNA in pAED4 vector⁷⁶ was used as the template, 5'-ACTCCATGGACGTGACCATCCAG-3' and 5'-CATATGTTACGACACGGGGATGG-3' were used as the pair of primers for the PCR reaction. The recombinant wt α A- and C-terminally truncated α A-crystallins were expressed in *Escherichia coli* and purified to homogeneity as described previously.⁷³ Recombinant wt α B-crystallin was expressed and purified as previously described.⁷⁷

Labeling of WT α -Crystallins and αA_{1-162} with Fluorescence Probes

To monitor the formation of hetero-oligomers between wt α -crystallins and αA_{1-162} , recombinant α -crystallins were first labeled with

fluorescence probes (lucifer yellow iodoacetamide [LYI] or 4-acetamid-4'-((iodoacetyl)amino)-stilbene-2,2'-disulfonic acid [AIAS]) as described previously.⁷⁸ Recombinant wt α A- and α B-crystallins, as well as αA_{1-162} solutions, were prepared in 20 mM MOPS buffer containing 100 mM NaCl, pH 7.9, at a concentration of 1 mg/mL. Solid AIAS or LYI was added to a final concentration of 4 and 10 mM, respectively, and the reaction was allowed to proceed for 12 hours at room temperature (23°C) in the dark. For labeling with LYI, the reaction was extended for another 6 hours at 37°C. Unreacted AIAS and LYI were separated from the fluorescently labeled α A-crystallins on a G-25 Sephadex desalting column equilibrated with 50 mM sodium phosphate buffer containing 100 mM NaCl, 2 mM dithiothreitol (DTT), pH 7.5.

Fluorescence Energy Transfer Assays

Fluorescence energy transfer was used to monitor subunit exchange or formation of hetero-oligomers. The reaction was initiated by mixing an equal volume of 0.5 mg/mL AIAS-labeled α A- or α B-crystallin and 0.5 mg/mL LYI-labeled αA_{1-162} at 37°C in 50 mM sodium phosphate buffer containing 100 mM NaCl and 2 mM DTT, pH 7.5. At time 0, 15, and 30 minutes, 20 μ L of the reaction mixture was taken and diluted 100 times with the same buffer. The emission spectrum of the sample excited at 335 nm was recorded using a Perkin-Elmer LS-5 spectrofluorometer (Perkin-Elmer, Waltham, MA). The decrease in fluorescence intensity at 415 nm of AIAS-labeled α -crystallin and the concomitant increase in fluorescence intensity at 525 nm of LYI-labeled α -crystallin were indicative of energy transfer owing to subunit exchange between the two labeled populations. Pilot experiments indicated that the subunit exchange reached equilibrium by 60 minutes of incubation.

Size Exclusion-Dynamic Light-Scattering Assay

To verify the formation of hetero-oligomers between wt α A-crystallin and αA_{1-162} , solutions of wt α A-crystallin and αA_{1-162} , as well as the 1:1 mixtures of wt α A-crystallin and αA_{1-162} , were analyzed by size exclusion chromatography with inline light-scattering, absorbance, and refractive index detectors.⁷⁴ A Superose 6HR 10/300 GL column (GE Healthcare, Piscataway, NJ) was connected in-line to an AKTA basic system (GE Healthcare), a Dawn-EOS multiangle laser light scattering detector (Wyatt Technology Corp., Santa Barbara, CA), and an Optilab-DSP refractive index detector (Wyatt Technology Corp.). Samples were loaded onto the column at a concentration of 1 mg/mL and eluted with 50 mM sodium phosphate buffer containing 100 mM NaCl, pH 7.0.

Labeling of α -Crystallins with Biotin and Avidin Pulldown Assays

The wt α -crystallin and αA_{1-162} solutions were prepared in PBS at a concentration of 2 mg/mL at pH 7.4. Sulfo-NHS-biotin stock solution at a concentration of 100 mM was made in dimethyl sulfoxide. To label wt α A-crystallin and αA_{1-162} with sulfo-NHS biotin, 5 μ L sulfo-NHS-biotin stock solution was added to 1 mL crystallin solutions and the reaction was carried out on ice for 2 hours with occasional shaking. The reaction was then terminated by adding lysine to a final concentration of 20 mM. Unreacted biotin was removed from the biotin-labeled crystallins using a G-25 Sephadex desalting column. To determine the formation of protein complexes, 100 μ g of biotin-labeled wt α A-crystallin and αA_{1-162} were mixed with an equal amount of unlabeled α -crystallin and incubated at 37°C for 30 minutes. The complexes were isolated by affinity pulldown using 50 μ L NeutrAvidin beads. After 5 washes with PBS, 1 mL/wash, the specifically bound proteins were retrieved from the beads using SDS-gel loading buffer, resolved by SDS-PAGE (15% gel), and stained with Coomassie blue.

Preparation of Lens Epithelial Cell Lysates

Human lens epithelial cells (SRA 01/04) were cultured using standard cell culture conditions (37°C, 5% CO₂, 10% fetal bovine serum) and

FIGURE 1. C-terminal truncation of α A-crystallin reduces subunit exchange between α A- and α B-crystallins. Recombinant wt α -crystallins and α A₁₋₁₆₂ were labeled with LYI or AIAS. FRET was used to determine the interaction between subunits of recombinant α -crystallins. A decrease in fluorescence intensity at 415 nm of AIAS-labeled α -crystallin with a concomitant increase in fluorescence intensity at 525 nm of LYI-labeled α -crystallin indicates subunits exchange. The data shown are representative of three reproducible independent experiments. (A) Subunit exchange between AIAS-labeled wt α A-crystallin and LYI-labeled wt α A-crystallin; (B) subunit exchange between AIAS-labeled α A₁₋₁₆₂ and LYI-labeled α A₁₋₁₆₂; (C) subunit exchange between AIAS-labeled wt α A-crystallin and LYI-labeled α A₁₋₁₆₂; (D) subunit exchange between AIAS-labeled wt α B-crystallin and LYI-labeled wt α A-crystallin; (E) subunit exchange between AIAS-labeled wt α B-crystallin and LYI-labeled α A₁₋₁₆₂.

collected by scraping at subconfluence. The cell pellets were homogenized with 50 mM Tris-HCl buffer containing 1 mM DTT, pH 7.6. After centrifugation at 100,000g for 10 minutes at 4°C, the supernatant was used as the source of the UPP for degradation assays.

Proteolytic Degradation Assay and Statistical Analysis

The wt α A-crystallins and α A₁₋₁₆₂ were labeled with ¹²⁵I by the chloramine T method.²⁶ Free ¹²⁵I was removed by Sephadex G25 desalting columns. The specific activity of ¹²⁵I-labeled proteins ranged from 0.2 to 0.5 μ Ci/ μ g. To determine the effects of hetero-oligomerization with wt α A- or α B-crystallin on degradation of α A₁₋₁₆₂, ¹²⁵I-labeled α A₁₋₁₆₂ was mixed with unlabeled wt α A- or α B-crystallins at the indicated ratios and incubated at 37°C for 30 minutes to form hetero-oligomers, and the mixtures were used for degradation assays. Unlabeled α A₁₋₁₆₂ was used as control to ensure each mixture contained the same amount of crystallins. To determine the effects of α A₁₋₁₆₂ on degradation of wt α A- or α B-crystallins, ¹²⁵I-labeled α A- or α B-crystallins were mixed with unlabeled α A₁₋₁₆₂ at the indicated ratios and incubated at 37°C for 30 minutes to allow the formation of hetero-oligomers. Unlabeled wt α A- or α B-crystallins were used as respective controls. Equal amounts of labeled substrates and unlabeled crystallins in each assay were used for the degradation assay (100 ng/assay). Degradation of the ¹²⁵I-labeled crystallins was determined as described by Huang et al.,³¹ using human lymphatic endothelial cell (HLEC) lysates as sources of ubiquitinating and proteolytic enzymes. Briefly, the proteolysis reaction mixture, in a final volume of 25 μ L, contained 50 mM Tris-HCl (pH 7.6), 5 mM MgCl₂, 1 mM DTT, 2 mM ATP, 10 mM creatine phosphate, 6 μ g of creatine phosphokinase, 2 μ g ubiquitin, 0.4 μ g recombinant Ubc4, and 15 μ L HLEC lysate (10 mg/mL protein). Ubc4 was expressed and purified essentially as described by Wing and Jain.⁷⁹ Degradation was initiated by addition of ¹²⁵I-labeled α A-crystallins (4 to 10 $\times$ 10⁴ cpm/assay) and the reaction mixtures were incubated at 37°C for 2 hours. Reactions were terminated by addition of 200 μ L of ice-cold 10 mg/mL bovine serum albumin,

immediately followed by 50 μ L of 100% trichloroacetic acid (TCA) (yielding a final concentration of 18.2% TCA), after which the samples were left on ice for 10 minutes. The extent of degradation was determined as the amount of TCA-soluble ¹²⁵I-labeled fragments of α A-crystallin. The total TCA-insoluble count at time 0 was defined as 100%. The percentage of degradation was proportional to the incubation time for the first 2 hours, thus we chose the 2-hour time point to compare the susceptibilities of substrates for degradation in this study. Because the substrate was not saturating and the degradation was expressed as percentage of the labeled substrate that was degraded during the 2-hour period, the susceptibility to degradation was not affected by variation concentrations of the labeled substrates under these experimental conditions. The portion of degradation that was inhibited in the presence of 20 μ M MG132, a potent proteasome inhibitor, was designated as UPP-mediated degradation. For both wt α -crystallin and α A₁₋₁₆₂, more than 85% of the degradation in the lysates was proteasome-dependent.

All experiments were performed in triplicate and repeated two to four times. For statistical analyses, data from several experiments were pooled and analyzed using the Student's *t*-test.

RESULTS

α A₁₋₁₆₂ Forms Hetero-oligomers with wt α A- and α B-Crystallins via Subunit Exchange

In the lens, α A-crystallin normally forms hetero-oligomers with α B-crystallin. To determine if α A₁₋₁₆₂ forms hetero-oligomers with wt α A- and α B-crystallins, we performed FRET assays using AIAS- and LYI-labeled wt α -crystallin or α A₁₋₁₆₂. As shown in Figure 1, AIAS-labeled α -crystallin exchanged subunits with LYI-labeled α -crystallin. The time-dependent decrease in fluorescence intensity at 415 nm of AIAS-labeled α -crystallin and the concomitant increase in fluorescence intensity at 525 nm of LYI-labeled α -crystallin indicate energy transfer between the two fluorescently labeled populations

FIGURE 2. C-terminally truncated αA -crystallin forms complexes with wt αA - and αB -crystallins. The wt αA -crystallin and αA_{1-162} were labeled with biotin, mixed with unlabeled α -crystallins and incubated at 37°C for 30 minutes. The complexes were isolated by affinity pulldown. The specifically bound proteins were retrieved from the beads, resolved by SDS-PAGE and stained by Coomassie blue. Lanes 1 and 2, biotin-labeled wt αA -crystallin and αA_{1-162} , respectively; Lane 3, pulldown of wt αA -crystallin with biotin-labeled αA_{1-162} ; Lane 4, pulldown of αA_{1-162} with biotin-labeled wt αA -crystallin; Lane 5, pulldown of wt αB -crystallin with biotin-labeled wt αA -crystallin; Lane 6, pulldown of wt αB -crystallin with biotin-labeled αA_{1-162} ; Lane 7, mock pulldown of unlabeled αA_{1-162} and wt αB -crystallin; lane 8, mock pulldown of unlabeled αA - and αB -crystallins.

owing to subunit exchange. Comparison of Figure 1B with Figure 1A showed that αA_{1-162} exchanged subunits similarly to that of wt αA -crystallin, but the efficiency of FRET of αA_{1-162} was lower in comparison with wt αA -crystallin, indicating the rate of subunit exchange of the αA_{1-162} was slower. As shown in Figure 1C, αA_{1-162} also exchanged subunits with wt αA -crystallin. Again, the efficiency of subunit exchange was lower as compared with the subunits exchange between the two differently labeled wt αA -crystallin populations (compare Fig. 1C with Fig. 1A). Consistent with the hetero-oligomers of αA - and αB -crystallins in the lens, wt αA -crystallin exchanged subunits readily with purified αB -crystallin as indicated by

FRET (Fig. 1D). αA_{1-162} also exchanged subunits with αB -crystallin, but at a reduced efficiency compared with wt αA -crystallin (Fig. 1, compare panel E with D).

To confirm that αA_{1-162} forms complexes with wt αA - or αB -crystallin, we labeled wt αA - or αA_{1-162} crystallins with biotin and then mixed them with unlabeled αA - or αB -crystallins. After incubation at 37°C for 30 minutes, the complexes were pulled down with NeutrAvidin beads and analyzed by SDS-PAGE. αA_{1-162} and wt αA -crystallin migrated as distinctive bands on SDS-PAGE (Fig. 2, compare lane 2 with lane 1). Biotin labeling slowed migration slightly, resulting in a smearing of the bands on SDS-PAGE, probably owing to heterogeneity of labeling. After incubation with biotin-labeled αA_{1-162} , unlabeled wt αA - or αB -crystallins were pulled down together with biotin-labeled αA_{1-162} (Fig. 2, lanes 3 and 6). Incubation with biotin-labeled wt αA -crystallin also resulted in pulling down of both unlabeled αA_{1-162} (Fig. 2, lane 4) and αB -crystallin (Fig. 2, lane 5). However, biotin-labeled wt αA -crystallin migrated together with αB -crystallin on SDS-PAGE (Fig. 2, lane 5). To ensure that the pulldown of unlabeled crystallins by NeutrAvidin beads was because of formation of hetero-oligomers with biotin-labeled αA -crystallins, we did a mock pulldown assay. In the absence of biotin-labeled αA -crystallins, only marginal levels of unlabeled αA - or αB -crystallins were pulled down (Fig. 2, lanes 7 and 8), indicating that the observed pulldown of unlabeled α -crystallins was because of formation of hetero-complexes between biotin-labeled and -unlabeled α -crystallins (Fig. 2, lanes 3–6).

Oligomers formed by purified C-terminally truncated αA -crystallins were smaller than those formed by purified wt αA -crystallin.⁷³ To verify that αA_{1-162} forms hetero-oligomers with wt αA -crystallin, we determined the sizes of oligomers of pure wt αA -crystallin, pure αA_{1-162} , and the 1:1 mixture of αA -crystallin and αA_{1-162} . Consistent with our previous findings, oligomers formed by pure αA_{1-162} were heterogeneous and in general smaller than those of pure wt αA -crystallin (Fig. 3). In

FIGURE 3. C-terminally truncated αA -crystallin forms oligomers with wt αA -crystallin. Oligomer states of wt (αA -crystallin [red]), αA_{1-162} (blue) and the 1:1 mixture of wt (αA -crystallin and αA_{1-162} [green]) were determined by size-exclusion chromatography coupled with multiangle light-scattering detectors. The sizes of oligomers formed by αA_{1-162} were smaller than those formed by wt αA -crystallin. High-mass aggregates were also detected in the preparation of αA_{1-162} , but not in the sample of wt αA -crystallin. The elution curve and sizes of oligomers of the 1:1 mixtures of wt αA -crystallin and αA_{1-162} were similar to those of wt αA -crystallin, except for the existence of high-mass aggregates of αA_{1-162} .

FIGURE 4. Oligomerization with wt αA - or αB -crystallins reduces the degradation of C-terminally truncated αA -crystallin. 125 I-labeled αA_{1-162} was mixed with indicated molar ratios of wt αA -crystallin (A) or wt αB -crystallin (B) to form complexes and then the mixtures were subjected to UPP-mediated degradation in lens epithelial cell lysates. The degradation of 125 I-labeled αA_{1-162} was monitored. The degradation assays were performed in triplicate and repeated four times. Data presented are means $\pm$ standard deviations. *Indicates $P < 0.05$ when compared with the degradation in the absence of wt αA - or αB -crystallins.

addition, a small fraction of αA_{1-162} , but not wt αA -crystallin, existed as high-mass aggregates. This is consistent with our previous finding that the αA_{1-162} was less thermally stable and prone to aggregation. The oligomer sizes of the 1:1 mixture of wt αA -crystallin and αA_{1-162} were slightly smaller than those of pure wt αA -crystallin, but were larger than those formed with pure αA_{1-162} (Fig. 3). The shape of the elution curve of the mixture was similar to that of pure wt αA -crystallin, except for the presence of high-mass aggregates of αA_{1-162} (Fig. 3). Similarly, the oligomer sizes of the 1:1 mixture of αA_{1-162} and wt αB were slightly smaller than those of pure wt αB , but were larger than those in the main peak of pure αA_{1-162} (Supplemental Fig. 1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-9147/-DCSupplemental>). Together, these data indicate that αA_{1-162} forms hetero-oligomers with wt αA - or αB crystallins. However, it appears that the aggregated form of αA_{1-162} was not incorporated into αA - or αB crystallins, as the proportions and sizes of the aggregates remained the same after incubation with wt αA -crystallin (Fig. 3 and Supplemental Fig. 1, available at <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-9147/-DCSupplemental>).

Oligomerization with WT αA - or αB -Crystallin Reduced the Degradation of αA_{1-162}

As we demonstrated previously, αA_{1-162} was more susceptible to degradation than wt αA -crystallin by the UPP. When incubated with lens epithelial cell lysates for 2 hours, approximately 4% of αA_{1-162} was degraded by the UPP (Fig. 4A), whereas only approximately 2% of the wt αA -crystallin

was degraded under the same condition (Fig. 5A). To determine the effects of formation of the hetero-oligomers with wt αA - or αB -crystallins on degradation of αA_{1-162} , 125 I-labeled αA_{1-162} was mixed with unlabeled wt αA - or αB -crystallins at 1:1 or 1:4 ratios and incubated at 37°C for 30 minutes to form hetero-oligomers. To rule out the possibility of substrate competition, unlabeled αA_{1-162} was used as a control for wt αA - or αB -crystallins. Incubation with wt αA -crystallin at both 1:1 and 1:4 ratios resulted in an approximately 40% decrease in degradation of αA_{1-162} (Fig. 4A), whereas incubation with wt αB -crystallin at the 1:4 ratio, but not at the 1:1 ratio, decreased the degradation of αA_{1-162} (Fig. 4B). These data indicate that formation of hetero-oligomers with wt αA - and αB -crystallins significantly reduces the degradation of αA_{1-162} (Fig. 4). The effects wt αA -crystallin on degradation of αA_{1-162} appeared to be stronger than those of wt αB -crystallin (Fig. 4, compare A with B).

Oligomerization with αA_{1-162} Altered the Degradation of WT αA - and αB -Crystallins

The above data indicate that oligomerization with wt αA - and αB -crystallins reduced the degradation of αA_{1-162} . To determine the reciprocal effects of αA_{1-162} on degradation of wt αA - or αB -crystallins, we incubated 125 I-labeled wt αA - or αB -crystallins with unlabeled αA_{1-162} at different ratios and then determined the degradation of 125 I-labeled wt αA - or αB -crystallins. The data in Figure 5A suggest that the presence of αA_{1-162} increased the degradation of wt αA -crystallin in a dose-dependent manner, although the increase was not statistically

FIGURE 5. Incorporation of C-terminally truncated αA -crystallin promotes the degradation of wt αA -crystallin but inhibits the degradation of wt αB -crystallin. 125 I-labeled wt αA - or 125 I-labeled wt αB -crystallin were mixed with αA_{1-162} or wt αA -crystallin at indicated molar ratios and incubated at 37°C for 30 minutes to form hetero-complexes. The mixtures were then subjected to degradation in lens epithelial cell lysates. The degradation of 125 I-labeled wt αA - or αB -crystallins was monitored. (A) Effects of αA_{1-162} on degradation of 125 I-labeled wt αA -crystallin; (B) effects of αA_{1-162} on degradation of 125 I-labeled wt αB -crystallin; (C) effects of wt αA -crystallin on degradation of 125 I-labeled wt αB -crystallin. The degradation assays were performed in triplicate and repeated three times (A, B) or two times (C). Data presented are means $\pm$ standard deviations. *Indicates $P < 0.05$ when compared with the degradation in the absence of αA_{1-162} .

significant. In contrast, incubation with αA_{1-162} reduced the degradation of wt αB -crystallin in a dose-dependent manner (Fig. 5B). To determine whether the inhibitory effects on degradation of wt αB -crystallin were specific to αA_{1-162} , we incubated ^{125}I -labeled wt αB -crystallin with the same amount of unlabeled wt αA -crystallin. As shown in Figure 5C, incubation with wt αA -crystallin had no detectable effects on degradation of wt αB -crystallin. Together, these data indicate that oligomerization with αA_{1-162} differentially affects the degradation of wt αA - and αB -crystallins.

DISCUSSION

We and others have demonstrated that the UPP is an important protein quality control system and that various forms of damaged or abnormal protein are selectively degraded by the UPP.^{25,27,29,31,73,80-83} However, it remains largely unknown whether formation of hetero-complexes with normal proteins alters the degradation behaviors of damaged proteins. Because most proteins in the lens exist as complexes, investigating the impact of formation of hetero-complexes on degradation of damaged proteins by the UPP would provide more physiologically relevant information regarding the role of the UPP in lens protein quality control. In this study, we examined the effects of formation of hetero-oligomers with wt α -crystallins on degradation of C-terminally truncated αA -crystallins using αA_{1-162} as a model substrate. The data show that αA_{1-162} readily interacts with wt αA - or αB -crystallins and forms hetero-oligomers with wt αA - or αB -crystallins. Consistent with our previous work,⁷³ αA_{1-162} was more susceptible than wt αA -crystallin to proteasome-mediated degradation. Oligomerization with wt αA - or αB -crystallins reduced the degradation of αA_{1-162} . Conversely, the presence of αA_{1-162} in the hetero-complexes enhanced the degradation of wt αA -crystallin, but reduced the degradation of wt αB -crystallin. These results suggest that formation of hetero-complexes not only alters the degradation behavior of αA_{1-162} , but also the degradation behaviors of wt α -crystallins.

α -Crystallins are the major components of the ocular lens and they play essential roles in maintaining lens transparency, both as structural proteins and as molecular chaperones.^{74,84} Mutation and adverse modifications of α -crystallins are associated with cataractogenesis.^{3,5,77,85-90} C-terminal truncation of αA -crystallin is one type of posttranslational modification that is associated with cataract.^{46,51,68,70} Modification of α -crystallins not only alters their chaperone activity,^{3,5,77,86-90} but also their stability and susceptibility to proteolytic degradation. For example, αA_{1-162} is thermally unstable and prone to aggregations.⁷³ If not removed in a timely manner from the lens, accumulation and precipitation of this modified crystallin would cause light scattering and lens opacity. We showed that isolated αA_{1-162} was more susceptible than wt αA -crystallin to proteasome-mediated degradation.⁷³ The preferential degradation of αA_{1-162} by the UPP may play a role in preventing its accumulation in the lens. Accumulation of αA_{1-162} and other forms of modified proteins in cataractous lenses may be related to impairment of UPP or attributable to altered susceptibility of the modified proteins to proteasome-mediated degradation. Consistent with this hypothesis, experimental impairment of the UPP by targeted expression of dominant negative ubiquitin in the lens results in accumulation of truncated crystallins and cataract.⁴⁵ We also found that glycation of αA_{1-162} reduced its susceptibility to proteasome-mediated degradation.⁴⁴ The reduced susceptibility of glycated proteins to proteasome-mediated degradation may explain why

αA_{1-162} was detected in diabetic lenses, but not detected in normal lenses.^{68,70}

In the lens, α -crystallins normally exist as hetero-oligomers.⁷⁴ C-terminally truncated crystallins also form hetero-oligomers with wt α -crystallins.⁹¹ Damaged proteins in the lens may form complexes with wt crystallins via subunit exchange or through chaperone functions of α -crystallins. Data from this work indicate that αA_{1-162} readily exchanges subunits with wt αA - and αB -crystallins, but the efficiency of subunit exchanges was lower compared with wt αA -crystallin (Fig. 1). This was consistent with previous findings.⁷⁵ αA_{1-162} is thermally unstable and prone to form high-mass aggregates. Dynamic light-scattering analysis shows that a significant portion of αA_{1-162} exists as high-mass aggregates (Fig. 3). It is plausible that the aggregated form of αA_{1-162} is not competent to subunit exchange, thus reducing the efficiency of subunit exchange, as we observed that the proportion and sizes of high-mass aggregates of αA_{1-162} remained the same after incubation with wt αA -crystallin (Fig. 3).

Our data show that formation of hetero-oligomers with wt αA - or αB -crystallin significantly reduced the degradation of αA_{1-162} (Fig. 4); however, the mechanisms by which oligomerization with wt αA - or αB -crystallin reduces the degradation of αA_{1-162} remain to be elucidated. We hypothesize that the enhanced degradation of αA_{1-162} is because of subtle conformation changes that result in the exposure of intrinsic degradation signals, such as hydrophobic patches.⁷³ The presence of wt αA - or αB -crystallin in the hetero-oligomers may partially mask the degradation signal on αA_{1-162} and reduce its degradation.

Interestingly, we found that incorporation of αA_{1-162} into oligomers of wt αA - or αB -crystallins also altered the degradation behaviors of wt αA - and αB -crystallins (Fig. 5), but in different manners. Whereas incorporation of αA_{1-162} increased the degradation of wt αA -crystallin, the presence of αA_{1-162} decreased the degradation of wt αB -crystallin. The different effects of αA_{1-162} on degradation of wt αA - and αB -crystallins suggest different models of recognition and degradation. We propose that the hetero-oligomers formed between αA_{1-162} and wt αA -crystallin, rather than their individual subunits, are recognized and degraded by the UPP. Formation of hetero-oligomers with wt αA -crystallin may partially mask the degradation signals of αA_{1-162} and reduce its degradation. On the other hand, if degradation signals on αA_{1-162} in the hetero-oligomers were recognized by the UPP, wt αA -crystallin subunits in the hetero-oligomers would be degraded together with αA_{1-162} (Fig. 6A). Thus, incorporation of αA_{1-162} into oligomers of wt αA -crystallin promotes the degradation of wt αA -crystallin. Consistent with this hypothesis, the degradations of αA_{1-162} and wt αA -crystallin in the hetero-complexes were comparable (compare Fig. 4A with Fig. 5A).

However, the above model cannot explain why the presence of αA_{1-162} reduced the degradation of wt αB -crystallin (Fig. 5B). We found that αB -crystallin is a better substrate than αA -crystallin for UPP-mediated degradation (compare Fig. 5B with Fig. 5A), indicating that wt αB -crystallin may have exposed signals for degradation by the UPP. Formation of hetero-oligomers with αA_{1-162} may mask the degradation signals on αB -crystallin and reduce its degradation (Fig. 6B). If that is the case, the interaction between wt αB -crystallin and αA_{1-162} must be different from the interaction between wt αB - and wt αA -crystallins, as the presence of wt αA -crystallin had no effects on degradation of wt αB -crystallin (Fig. 5C).

αB -crystallin is a chaperone and it actively interacts with proteins with abnormal structures and protects them from aggregation and precipitation. The interaction between wt αA -crystallin and wt αB -crystallin is mainly through subunit

FIGURE 6. Hypothetic models for the effects of wt α A- and α B-crystallins on degradation of C-terminally truncated α A₁₋₁₆₂-crystallin. (A) wt α A-crystallin is resistant to degradation because of the lack of degradation signals on the surface. C-terminal truncation results in conformational changes and exposure of degradation signal, such as hydrophobic patches (marked red in the diagram) and render α A₁₋₁₆₂ more susceptible to degradation by the UPP. Oligomerization with wt α A-crystallin masks some of the degradation signals on α A₁₋₁₆₂, thus reducing its degradation. However, any unmasked degradation signals on α A₁₋₁₆₂ subunits in the hetero-oligomers also bring the wt α A-crystallin subunits in the oligomers for degradation. Thus, oligomerization with α A₁₋₁₆₂ increases the degradation of wt α A-crystallin. (B) wt α B-crystallin is susceptible to degradation because of the availability of hydrophobic patches on the surface (marked dark red in the diagram). The hydrophobic patches serve both as degradation signals and as client-binding sites for its chaperone function. The degradation signal on α A₁₋₁₆₂ may overlap or share with chaperone-binding signal. In addition to subunit exchange, oligomerization between wt α B and α A₁₋₁₆₂ may involve chaperone-client binding, which masks the degradation signals on both wt α B-crystallin subunits and α A₁₋₁₆₂ subunits. Thus, the susceptibilities of both wt α B-crystallin and α A₁₋₁₆₂ in the hetero-oligomers are reduced.

exchange; however, the interactions between α A₁₋₁₆₂ and wt α B-crystallin may involve chaperone-client binding, in addition to subunit exchange (Fig. 6B). Hydrophobic interaction is often involved in binding of client proteins by chaperones,^{92,93} and hydrophobic patches on protein surface are also signals for UPP-mediated degradation.^{29,73,83,94-97} The chaperone-client interaction between wt α B-crystallin and α A₁₋₁₆₂ may mask the degradation signal on α B-crystallin and reduce its degradation by the UPP.

Our data obtained from cell-free experiments show that recombinant α B-crystallin is degraded faster than recombinant α A-crystallin, even faster than C-terminally truncated α A-crystallin. The greater susceptibility of α B-crystallin to UPP-mediated degradation raises the question why the native α B-crystallin is not cleared in the lens, as there is an active UPP in lens fibers. This inconsistency could be because of potential difference in regulation and selectivity of the UPP between intact lens and lens epithelial cell lysate. This difference might explain why native α B-crystallin in intact lenses is not cleared, although recombinant wt α B-crystallin is rapidly degraded by the UPP in the cell-free system. Another possibility is that the conformation of the recombinant wt α B-crystallin might not be identical to that of native α B-crystallin in the intact lens. The

subtle difference in conformation might contribute to the difference between recombinant wt α B-crystallin and native α B-crystallin in susceptibility to UPP-mediated degradation. Furthermore, native α B-crystallin in the lens is normally associated with α A-crystallin and other client proteins. The binding of client proteins (including truncated α A-crystallin) to α B-crystallin in the lens might mask the degradation signals in α B-crystallin and prevent it from degradation, as illustrated in Figure 6B.

In summary, this study confirmed the previous finding that α A₁₋₁₆₂ is preferentially degraded by the UPP. Formation of hetero-oligomers with wt α A- or α B-crystallins reduces, but does not prevent, the selective degradation of α A₁₋₁₆₂. Furthermore, the presence of α A₁₋₁₆₂ also affects the degradation of wt α A- and α B-crystallins. Future structural characterization of the hetero-oligomers formed by α A₁₋₁₆₂ and wt α A- or α B-crystallins will help elucidate mechanism by which the presence of α A₁₋₁₆₂ alters the degradation of wt α A- and α B-crystallins.

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