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Peptide-induced formation of protein aggregates and amyloid fibrils in human and guinea pig α A-crystallins under physiological conditions of temperature and pH

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Abstract

α A^{66–80} peptide forms with age in the lens nucleus of humans and guinea pigs, and binds to α A-crystallin by hydrophobic bonds to produce protein aggregates. The purpose of the present study was to use negative-stain transmission electron microscopy (TEM) to investigate peptide/crystallin structures that form when α A^{66–80} peptide is incubated with recombinant human (HU) and guinea pig (GP) α A-crystallin, and GP α A^{ins}-crystallin, under physiological conditions of temperature and pH. HU and GP α A-crystallin each consists of 173 amino acid residues with only eight differences. GP α A^{ins}-crystallin contains an insertion of 23 amino acids into the sequence for normal GP α A-crystallin. Synthetic α A^{66–80} peptide was incubated with each crystallin for 24 hrs in phosphate buffered saline at 37° C, pH 7.4. Samples were loaded onto electron microscopy grids and analyzed by TEM. With GP α A-crystallin, the α A^{66–80} peptide appeared to first form up to 700 nm long, independent peptide fibrils, which subsequently bound numerous α A-crystallin oligomers along the entire length of the fibril to form up to 30 nm thick peptide/oligomer fibril structures. In contrast, HU α A-crystallin somehow prevented independent α A^{66–80} peptide fibrillation, and instead joined with the peptides to form amorphous aggregates and linear chains of α A oligomers. When incubated alone, GP α A^{ins}-crystallin eventually lost all oligomeric

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Author contributions

All authors participated in the research and approved the final version of the manuscript. FJG supervised the project and prepared the manuscript. AK and SJ contributed equally to the study and were involved in the experimental design, performance of experiments, discussion of results and preparation of the manuscript. VAK supervised the TEM analysis. Undergraduate students JC, AP and ES helped to produce and purify the recombinant proteins used in the study, and participated in discussion of experimental results.

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Competing Interests

The authors declare no competing interests.

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structure and formed amorphous aggregates. However, addition of αA^{66-80} peptide to the incubation medium resulted in the formation of highly-organized peptide/ αA^{ins} oligomer amyloid fibrils, nearly 400 nm in length, with oligomer dimers appearing to stack one on top of another. Mature peptide/ αA^{ins} oligomer fibrils were highly compressed and 40% thicker than observed protofibrils (17 nm vs. 12 nm thickness). Use of a control V72P αA -crystallin peptide, as well as addition of the disulfide bond reducing agent DTT to the incubation medium, prevented fibril formation. Turbidity and Thioflavin T fluorescence analyses confirmed the observed peptide-induced fibril formation. This study represents the first time that fibrillation of αA -crystallin has been accomplished under physiological conditions of temperature and pH without use of denaturants. Fibrillation of GP αA^{ins} -crystallin was linked with possible intermolecular disulfide-crosslinking of oligomers. The finding that αA^{66-80} peptide induces aggregate formation with HU αA -crystallin supports a role for this peptide in the development of HU nuclear cataract.

Graphical Abstract

Keywords

lens crystallins; human; guinea pig; nuclear cataract; αA^{66-80} peptide; transmission electron microscopy; amyloid fibrils; disulfide crosslinking

1 Introduction

A hallmark of the aging human (HU) lens nucleus is the formation of large, abnormal protein aggregates that cause increased lens nuclear light scattering (NLS), leading eventually to nuclear cataract (Datiles et al., 2008; Jedziniak et al., 1973; Thurston et al., 1997). In this regard, nuclear cataract is one of approximately 40 “protein conformational diseases” (e.g., Alzheimer’s disease and Huntington’s disease) in which proteins undergo abnormal unfolding followed by aggregation (Chiti and Dobson, 2006; Harding, 1998). Protein aggregates of a size similar to those associated with HU nuclear cataracts have also been detected in the lens nucleus of guinea pigs (GP’s) treated with hyperbaric oxygen (HBO) (Simpanya et al., 2005). Although age-related lens crystallin aggregation has been studied for many years (Jedziniak et al., 1973; Spector and Roy, 1978), its mechanism of formation is still not well-understood. The high molecular weight (HMW) fraction of HU lenses also contains large amounts of degraded/truncated crystallins with MW’s <18,000 Da (Horwitz et al., 1983; Srivastava, 1988; Srivastava et al., 1996). Imaging mass spectrometry has revealed in the aging HU lens nucleus the presence of multiple truncation products of α -crystallin (Grey and Schey, 2009), with crystallin fragments beginning to appear at early middle age (Su et al., 2010). Convincing evidence has been gathered in recent years

indicating that an interaction of low molecular weight crystallin-derived peptides <3,500 Da with intact crystallins may be a key event in age-related protein aggregation occurring in the lens nucleus (Sharma and Santhoshkumar, 2009).

A major age-related lens peptide found at elevated concentrations in HU nuclear cataracts is the α A^{66–80} peptide ⁶⁶SDRDKVFIFLDVKHF⁸⁰ possessing a MW of 1,866 Da (Santhoshkumar et al., 2011; Santhoshkumar et al., 2008). This peptide, which contains a portion of the α A-crystallin chaperone site (residues 70–80) (Sharma et al., 2000), has also been detected in the lenses of old GP's, and has been observed to increase in concentration following treatment of the animals with HBO (Raju et al., 2015). The α A^{66–80} peptide consists of 15 amino acids, 7 of which are hydrophobic (Fig. 1). *In vitro* studies have shown that the peptide can bind to both HU and GP α A-crystallin by hydrophobic bonds to produce “hydrophobic patches” which attract additional crystallins to form HMW aggregates (Kannan et al., 2013; Raju et al., 2015; Santhoshkumar et al., 2011). In addition, the α A^{66–80} peptide is known to possess properties similar to those of β -amyloid peptide, which produces amyloid fibril formation in Alzheimer's disease (Balbach et al., 2000; Raju et al., 2017). The ⁷⁰KFVIF⁷⁴ sequence present in the core of the peptide (Fig. 1) is similar to the ¹⁶KLVFF²⁰ region of β -amyloid peptide, enabling the peptide to form amyloid fibrils (Kannan et al., 2014; Santhoshkumar et al., 2011), as well as generate H₂O₂ (Raju et al., 2017), when incubated *in vitro*.

It has been known for over a decade that under certain denaturing conditions (e.g., low pH or in the presence of guanidine HCl at 60°C), each of the lens α A-, α B-, β - and γ - crystallins, as well as aged HU lens crystallin fragments, can assemble into amyloid fibrillar structures (Ghaffari Sharaf et al., 2017; Kosinski-Collins and King, 2003; Meehan et al., 2004; Meehan et al., 2007; Papanikolopoulou et al., 2008; Sharma and Ghosh, 2017; Srivastava et al., 2017). Many, perhaps all, polypeptide chains are believed to have a propensity to self-assemble into amyloid fibrils under appropriate conditions (Chiti and Dobson, 2006). The central, β -sheet structured domain of α -crystallin is believed to be highly susceptible to amyloid fibrillation (Carver et al., 2017). UV light also has been shown to induce *in vitro* the formation of highly ordered protein β -sheets in γ D-crystallin, typical of β -amyloid secondary structure (Cetinel et al., 2017; Moran et al., 2013; Zhang et al., 2017). However, for some reason, fibrils have not been observed to be present in either normal HU lenses or HU maturity-onset cataracts (Harding, 1972; Meehan et al., 2004). It is possible that the environment of the human lens, including perhaps the chaperone-like function of α -crystallin (Ecroyd and Carver, 2009; Horwitz, 1992), may act to inhibit crystallin fibril formation.

α -crystallin is the major protein in the lens, accounting for approximately 40% of the total protein content (Horwitz et al., 1999). In the intact lens, α -crystallin consists of both α A and α B subunits at a ratio of 3:1. The α -crystallin complex ranges between 600 to 900 kDa in molecular weight, being comprised of between 30 and 45 subunits, although studies conducted with both bovine α B-crystallin (Aquilina et al., 2003) and individual recombinant HU α A- and α B-crystallins (Pescek et al., 2009) have indicated a polydispersity of oligomeric forms. Other studies have suggested that α A-crystallin may play a relatively more important role than α B in maintaining lens transparency since a knockout of α A-

crystallin in the mouse lens lead to severe cataract, while deletion of α B- crystallin produced no lens phenotype (Brady et al., 1997; Brady et al., 2001). As mentioned above, α A-crystallin is capable of forming amyloid fibrils under denaturing conditions, and the age-related α A-crystallin fragment α A^{66–80} peptide can bind to α A- crystallin to produce HMW aggregates, as well as form amyloid fibrils independently. All of this information emphasizes the need for conducting a detailed investigation and characterization of the interaction of α A^{66–80} peptide with α A-crystallin oligomers, as we have done in the current study with the use of negative-stain transmission electron microscopy (TEM), a common method for visualizing amyloid fibrils *in vitro* (Meehan et al., 2004; Tosoni, 2011).

In the present work, we have investigated possible fibril formation coincident with the binding of α A^{66–80} peptide to HU and GP α A-crystallin, as well as to GP α A^{ins}-crystallin. Bovine γ D-crystallin was employed as a control crystallin that does not bind α A^{66–80} peptide in a specific manner. The studies have been conducted under physiological conditions of pH and temperature in contrast to the majority of other fibril studies which have employed either denaturants or extremes of pH, temperature or ionic strength (Yang et al., 2015). HU and GP α A-crystallin each contain 173 amino acids with eight amino acid differences between the two sequences (Fig. 1). One potentially important difference is the presence of two cysteine –SH groups in the sequence of HU α A-crystallin, compared to just one in the GP protein. α A^{ins}-crystallin is a protein produced by evolutionary alternative splicing which exists in the lenses of a number of rodent species including rat, mouse, and pika, and a few non-rodent mammals such as GP, kangaroo, hedgehog and bat, but not in species such as rabbit, dog and primates (Hendriks et al., 1988; van Dijk et al., 2001). α A^{ins}-crystallin mRNA has been found in both the epithelium and fibers of mouse lenses at levels of 10 to 20 percent of total α A-crystallin mRNA (King and Piatigorsky, 1984). GP α A^{ins}-crystallin contains an insertion of 23 amino acids between residues 63 and 64 to produce a total of 196 amino acids, compared to 173 for the normal crystallin (Fig. 1). It is unclear why α A^{ins}-crystallin has been retained in the lenses of certain species for over 70 million years of evolution, especially since the protein has been shown to possess reduced chaperone-like activity compared with that of normal α A-crystallin (Smulders et al., 1995).

As a control for the α A^{66–80} peptide, a V72P α A-crystallin peptide in which proline was substituted for valine at residue 72 was employed. Although the V72P peptide binds to some extent to α A-crystallin, it has been shown to lack the β -sheet structure of α A^{66–80} peptide, and thus does not form fibrils when incubated alone (Kannan et al., 2014). This study has demonstrated for the first time that α A^{66–80} peptide under non-denaturing conditions of temperature and pH is able to induce the formation of two very different types of fibrils in GP α A- and α A^{ins}-crystallins, while inducing amorphous aggregate formation in HU α A-crystallin.

2 Materials and methods

2.1 Materials

The two peptides employed in the study, α A^{66–80} peptide (SDRDKFVIFLDVKHF) and control V72P peptide (SDRDKFPVIFLDVKHF), were obtained at >95% purity from GenScript (Piscataway, NJ, U.S.A.). Bovine γ D-crystallin was obtained from Enzo Life

Sciences (Farmingdale, NY, U.S.A.). All other reagents were from Sigma-Aldrich (St. Louis, MO, U.S.A.) unless otherwise specified.

2.2 Creation of wild type and mutant HU and GP α A-crystallin

Wild type (WT) HU α A-crystallin of 173 amino acids was overexpressed using a pET23d expression vector (EMD Millipore, Novagen, Billerica, MA, U.S.A.). Mutant HU C142S α A-crystallin, with a substitution of one cysteine at residue 142, was created with primers designed by NEBaseChanger™, online software for primer design, and with a Q5 Site-Directed Mutagenesis Kit from New England Biolabs (NEB, Ipswich, MA, U.S.A.). The gene for GP α A^{ins}-crystallin, containing an insert of 23 amino acids in the region from residue 63 of WT GP α A-crystallin, was synthesized by Genscript (Piscataway, NJ, U.S.A.) and cloned into a pET28a expression vector between the *NcoI* and *XhoI* restriction sites. A plasmid system for expression of normal WT GP α A-crystallin of 173 amino acids was created by deletion of the 69 base pair insert in the GP α A^{ins}-crystallin gene with primers designed with NEBaseChanger™, and with use of a Q5 Site-Directed Mutagenesis Kit from NEB. An S142C mutant of GP α A-crystallin, containing two cysteines rather than the one present in the WT protein, was created in a similar manner. All plasmid constructs used were sequenced to confirm identity (Supplemental Fig. 1).

2.3 Overexpression and purification of GP and HU α A-crystallin

Plasmids coding for the corresponding five α A-crystallin clones, namely GP, mutant GP S142C, HU, mutant HU C142S and GP α A^{ins}, were transformed into *Escherichia coli* BL21 (DE3) cells for protein overexpression. Cells were grown at 37°C in Luria Bertani broth to an OD₆₀₀ of 0.6 – 0.8. Overexpression of the proteins was induced with 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG, EMD-Calbiochem La Jolla, CA, U.S.A.), and the cells were harvested 3 hrs later. Harvested bacterial pellets were suspended in Buffer A [20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5 mM Tris 2- carboxyethyl phosphine (TCEP), 5% (v/v) glycerol], frozen overnight at minus 80° C, and lysed by sonication. Cell debris was removed by centrifugation at 12,000 rpm (18,514g) for 30 min at 4° C. For GP α A^{ins}-crystallin, only the 10% of the protein that remained in the supernatant (the rest being present in the pellet) was purified and used for experiments. Efforts to unfold the WI crystallin in 6M urea and refold by dialysis against water or buffer were unsuccessful in solubilizing the protein, as had previously been done for rat α A^{ins}-crystallin (Smulders et al., 1995). The supernatant was loaded onto a Buffer A-pre- equilibrated Highprep-DEAE-Fast Flow resin column (GE Healthcare Bio-Sciences, Pittsburgh, PA, U.S.A.) and eluted with a gradient of Buffer B (20 mM Tris-Cl, pH 8.0, 1000 mM NaCl, 0.5 mM TCEP, 5% (v/v) glycerol). The eluted fractions were analyzed by 12% (w/v) SDS-PAGE and fractions containing prominent amounts of each protein of interest were pooled, concentrated using centrifugal filters, and loaded on to a Sephacryl S300 size exclusion chromatography column (GE Healthcare Bio-Sciences, Pittsburgh, PA, U.S.A.) that had been pre-equilibrated with phosphate buffered saline (PBS, 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl), pH 7.4. Eluted fractions from the size exclusion column containing the protein of interest were verified by 12% (w/v) SDS-PAGE. Fractions having maximum purity were pooled and their concentration determined using a Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL, U.S.A.).

2.4 HPLC and SDS-PAGE analysis

Purified recombinant α A-crystallin proteins were injected on to an HPLC system (Shimadzu Scientific Co., Kyoto, Japan) with a size exclusion column (Biosep-SEC- S4000; Phenomenex, Torrance, CA, U.S.A.) equilibrated with PBS, pH 7.4. The flow rate was 1 ml/min and the column was attached to a UVB (280 nm wavelength) detector. The column was calibrated with the following protein standards and molecular weights (MWs): Blue dextran (2000 kDa), thyroglobulin (660 kDa), apoferritin (443 kDa), β -amylase (200 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa). MWs of α A-crystallin oligomers were estimated from their HPLC elution times. The logs of the MW standards were plotted against elution time, and Excel was used to generate a linear trend line and corresponding equation. The peak elution time of each α A-crystallin oligomer was used in the equation to solve for kDa MW. Purified α A-crystallins were analyzed by 12% SDS-PAGE after reduction of disulfide in a 4x SDS gel sample buffer 0.5 M Tris/HCl, pH 6.8, containing 20% glycerol [vol/vol], 20% SDS, 1% bromophenol blue, and 50 mM DTT (Laemmli, 1970). Samples were boiled at 95 to 97 $^{\circ}$ C for 5 minutes and 10 μ g loaded into each well. The gel was run at a constant current of 20 mA until the bromophenol blue dye was a few millimeters from the end, followed by Coomassie Brilliant Blue staining. MW markers were from Gold Biotechnology, Inc. (St. Louis, MO, U.S.A.).

2.5 Prediction of secondary structures of α A-crystallin and peptides

Amino acid sequences of HU and GP α A-crystallin, as well as α A^{66–80} and V72P peptides, were submitted to an online server (I-TASSER; <http://zhanglab.ccmb.med.umich.edu/I-TASSER/>) to predict secondary structures, which were represented for visualization of β -sheets present in the structures. A Zyggregator (Tartaglia et al., 2008) program was employed for predicting aggregation and fibril-forming propensities of HU and GP α A-crystallin.

2.6 Transmission electron microscopy (TEM)

In order to visualize morphological effects on lens crystallins following incubation of the proteins with either α A^{66–80} or control V72P peptide, negative-stain TEM was employed. Protein (0.25 mg/ml) and peptide (0.05 mg/ml) were incubated together in PBS, pH 7.4, 20 μ l volume, 37 $^{\circ}$ C, for 24 hrs. At the end of the incubation period, 10 μ l of sample was loaded on to a 400 mesh Formvar carbon-coated copper electron microscopy grid (Electron Microscopy Sciences, Hatfield, PA, U.S.A) as described earlier (Raju et al., 2012) with slight modification. GP α A-crystallin, HU α A-crystallin, and GP α A^{ins}-crystallin were also incubated alone for 0 and 24 hrs, and analyzed by TEM. Zero time samples were centrifuged first [14,000 rpm (20,817g) for 10 min], but 24 hr samples were not. After loading each sample, the grid was washed with 10 μ l of deionized water, negatively stained with 10 μ l of 2% w/v aqueous uranyl acetate, and then washed again with 10 μ l of deionized water. After 1 hr, samples were viewed with use of an FEI Morgagni 268 TEM at either 80 or 100 kV. For determination of fibril and oligomer diameters, an ImageJ software package was employed (Schneider et al., 2012). Diameters of light areas were measured at the inner borders of darkly stained regions which surrounded the electron lucent structures of interest. Commands employed were the straight line tool and analyze/measure function. Data were

calibrated using scale bars (nm) on the images with the analyze/set scale function of ImageJ. Measurements were collated and analyzed using Microsoft Excel for mean and standard deviation (S.D.).

2.7 Turbidity and supernatant protein analysis

Turbidity analyses were conducted at a wavelength of 400 nm to evaluate peptide, crystallin, and crystallin/peptide aggregation and fibril formation *in vitro*. Briefly, a 100 μ l mixture of protein (0.25 mg/ml) and peptide (0.05 mg/ml) in PBS, pH 7.4, 37° C, at 0 and 24 hr incubations were added to a 96-microwell plate, and absorbance measured at 400 nm wavelength using a BioTek Cytation 3 reader (BioTek Instruments, Inc., Winooski, VT, U.S.A.). Each sample mixture was centrifuged at 14,000 rpm (20,817g) for 10 min, and the supernatant analyzed for protein concentration using a Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL, U.S.A.). Results were expressed as the mean $\pm$ S.D. for three separate experiments.

2.8 Thioflavin T fluorescence

Thioflavin T (ThT) fluorescence was measured using an RF-6000 Shimadzu spectrofluorophotometer (Shimadzu, Columbia, MD, U.S.A.). ThT is an amyloid detection dye whose fluorescence increases after binding to amyloid fibrillary aggregates. In order to assess fibril formation, 20 μ l aliquots of samples were taken from 0 and 24 hr incubations of proteins (0.25 mg/ml) and peptides (0.05 mg/ml) and mixed with ThT to a final concentration of 16 μ g/ml. Fluorescence emission spectra were measured at a wavelength range of 450 to 500 nm (445 nm excitation). An increase in fluorescence emission intensity at 486 nm (Wang et al., 2010) was taken as an indication of amyloid fibril formation.

2.9 Statistics

Numbers of biological repeats for the experiments are indicated in the figure legends. Data for the biological assays of Figs. 4 and 8 represent results of three independent biological repeats with means $\pm$ S.D. being presented. TEM micrographs present in Figs. 3, 5, 6 and 7 are representative of 2–3 experiments each. Thicknesses of fibrils present in Figs. 5C and 7C were measured in 2–4 micrographs of one experiment each.

3 Results

3.1 HPLC and SDS-PAGE analysis

Four of the five purified recombinant α A-crystallins investigated in the study (GP, mutant GP S142C, HU and mutant HU C142S) eluted at about the same time period during HPLC analysis (each peak at approximately 9 min) (Fig. 2A). A linear fit of the data to MW standards yielded peak MWs of 496 and 486 kDa for GP and HU α A-crystallin, respectively, corresponding to 25 and 24 subunits per oligomer. In contrast, purified recombinant GP α A^{ins}-crystallin lost its organized oligomer structure during passage through the HPLC column, exhibiting mainly monomers with some higher MW species (Fig. 2A). Results of SDS-PAGE analysis of the five purified recombinant α A-crystallins are shown in Fig. 2B. The MW of α A^{ins}-crystallin is 22,476 Da, which compares to about 20,000 Da for the other α A-crystallins.

3.2 TEM analysis of αA^{66-80} and V72P peptides

TEM analysis was conducted to determine whether αA^{66-80} and control V72P peptides would form amyloid fibrils under the experimental conditions employed in the current study. I-TASSAR analysis indicated the presence of β -sheet structure for αA^{66-80} but not V72P peptide (Fig. 3A). The peptides (0.05 mg/ml) were incubated separately in PBS, pH 7.4, at 37°C. After 24 hrs, αA^{66-80} peptides had formed long, rigid fibrils of up to 1 μ m in length (Fig. 3B). Addition of 50 mM DTT to the incubation medium resulted in the production of αA^{66-80} peptide fibrils similar to those formed in the absence of DTT, with no major structural differences (Fig. 3C). In contrast, V72P peptides showed no evidence of fibril formation after 24 hrs of incubation (Fig. 3D).

3.3 Turbidity and supernatant analysis

Turbidity analysis was conducted at a wavelength of 400 nm in order to evaluate peptide, crystallin, and crystallin/peptide aggregation and fibril formation at 0 and 24 hrs of incubation (Fig. 4A). This type of analysis is frequently employed for assessing the extent of protein aggregation and amyloid fibril growth (Chaudhuri et al., 2014; Hall et al., 2016; Zhao et al., 2016). αA^{66-80} peptide, as well as recombinant GP and HU αA -crystallin, each showed only slight turbidity at 0 and 24 hrs when incubated alone (Fig. 4A). In contrast, when the peptide was mixed with either of the two crystallins, turbidity was observed to increase significantly at 0 time ($p < 0.05$), and to increase further after 24 hrs ($p < 0.01$). GP αA^{ins} -crystallin by itself exhibited considerable turbidity at 0 time, and this increased three-fold at 24 hrs (Fig. 4A). The 0 time value more than doubled following the addition of αA^{66-80} peptide ($p < 0.05$), and although the 24 hr value increased by more than 50%, the increase was not statistically significant compared to that for protein alone. Incubation of the αA^{66-80} peptide with γD -crystallin showed only slight turbidity, which may have been the result of self-aggregation of the peptide. Similarly, incubation of control V72P peptide with either GP or HU αA -crystallin, or with GP αA^{ins} -crystallin, produced an insignificant elevation in turbidity at the two time periods, compared with values for protein alone (Fig. 4A).

Following turbidity analysis, each solution was centrifuged and the concentration of protein remaining in the supernatant was measured in order to determine the percentage of soluble protein that had aggregated and precipitated. GP αA -crystallin plus αA^{66-80} peptide incubated for 24 hrs showed a 12% loss of WS protein compared to incubation of the protein alone ($p < 0.05$). However, loss of HU αA -crystallin when incubated with the peptide for the same time period was not statistically different from the value for the crystallin alone (Fig. 4B). Loss of GP αA^{ins} -crystallin after a 24 hr incubation with αA^{66-80} peptide amounted to nearly 60% compared to incubation of crystallin alone ($p < 0.05$). Incubation of bovine γD -crystallin with αA^{66-80} peptide, or incubation of each of the three αA -crystallins with V72P peptide for 24 hrs produced no significant decreases in WS protein levels compared with results for protein alone (Fig. 4B).

3.4 TEM analysis of recombinant GP αA -crystallin incubated with peptides

Results of studies described above indicated that αA^{66-80} peptide binds to GP αA -crystallin *in vitro* to produce protein aggregates, some of which become WI (Figs. 4A and

B). In order to view the aggregates and determine whether formation of either amorphous aggregates or ordered fibrils had occurred, TEM analysis was conducted. The protein was incubated in PBS at 37° C, with and without αA^{66-80} or V72P peptide, and aliquots of the mixtures were spotted on Formvar and carbon-coated copper grids, and analyzed. Incubation of GP αA -crystallin by itself for either 0 or 24 hrs showed the presence of independent αA -crystallin oligomers with an average diameter of 10 $\pm$ 3 nm (Figs. 5A and B, respectively). The oligomers possessed a well-defined shape and were homogeneously distributed. Based on the results of HPLC analysis (Fig. 2A), the average recombinant GP αA oligomer would consist of 25 subunits with a MW of 496 kDa. Incubation of αA^{66-80} peptide with GP αA -crystallin protein for 24 hrs showed the presence of independent αA -crystallin oligomers (Fig. 5C, large arrows without tails) along with what appeared to be a nearly 300 nm long, 9 $\pm$ 3 nm thick, peptide fibril (Fig. 5C, arrowhead, #1), similar to the αA^{66-80} peptide fibril shown in Fig. 3B. In addition, three apparent peptide fibrils with bound αA -crystallin oligomers, 200–300 nm in length, with varying thicknesses of 25 $\pm$ 11 nm, 11 $\pm$ 3 nm and 21 $\pm$ 8 nm, can be observed (Fig. 5C, arrows with tails, #'s 2, 3 and 4, respectively). Nineteen additional fibrils were measured in three additional micrographs from the experiment of Fig. 5C, and the thicknesses ranged from 5 to 75 nm. It appears that peptide fibrils may have formed first, followed by the binding of GP αA -crystallin oligomers to form increasingly thick peptide/crystallin oligomer fibrils. This type of scenario is suggested by the varying thicknesses of fibril #2 in Fig. 5C, which range from 12 to 40 nm. Additional TEM evidence for this type of mechanism in which the peptide fibril forms first is contained in Supplemental Fig. 2A which shows a highly tapered αA^{66-80} peptide/GP αA oligomer fibril that is nearly 700 nm long (fibril #2). While the base of the fibril is only 2.4 nm thick, and presumably consists of bare peptide fibril, the upper portion is 21 nm thick and represents peptide fibril with bound αA oligomers. Apparent gaps in fibrils located between bound oligomers are also evident (Supplemental Figs. 2 A–E), suggesting a random binding of oligomers. A few possible disulfide cross-linked oligomers can be seen in Figs. 5C and 7G (small arrows). A possible scheme for the binding of GP αA -crystallin oligomers to αA^{66-80} peptide fibrils is shown in Fig. 5D. In contrast to the results shown in Fig. 5C, incubation of V72P peptide with the crystallin for 24 hrs produced no evidence of any fibril formation (Fig. 5E). Incubation of αA^{66-80} peptide with bovine γD -crystallin for 24 hrs also produced no type of aggregate (Fig. 5F), compared with the control (Fig. 5G).

In view of the fact that intermolecular protein disulfide has been linked with amyloid fibril formation (Herrmann and Caughey, 1998; Welker et al., 2001), an experiment was conducted in which the disulfide bond reducing agent dithiothreitol (DTT) was incubated with αA^{66-80} peptide and GP αA -crystallin for 24 hrs. TEM analysis indicated that under these conditions, extensive binding of αA -crystallin oligomers to independent αA^{66-80} peptide fibrils was not observed (Fig. 5H), as it had been without use of DTT (Fig. 5C). While a few apparent thin peptide fibrils are visible in Fig. 5H (arrows), addition of DTT prevented the formation of peptide/ αA -crystallin oligomer fibrils. To test whether just a single crystallin –SH group in GP αA -crystallin might be required for fibril formation, mutant GP S142C αA -crystallin (with two –SH groups instead of one) was incubated with αA^{66-80} peptide for 24 hrs. TEM analysis indicated that under this condition, the peptide was not able to induce amyloid fibril formation with the crystallin (Fig. 5I). Two other GP

α A-crystallin mutants, A13T and G153T, were also tested for their ability to form fibrils when incubated with α A^{66–80} peptide. TEM analysis for each of the mutants also showed no evidence of fibril formation after 24 hrs of incubation with the peptide (Supplemental Fig. 3).

3.5 TEM analysis of recombinant HU α A-crystallin incubated with peptides

HU α A-crystallin, with an amino acid sequence differing from the GP protein by only eight residues (Fig. 1), also exhibited protein aggregation when incubated with α A^{66–80} peptide (Fig. 4A). In order to determine the nature of the aggregates, TEM analysis was conducted. Incubation of the crystallin by itself for 0 and 24 hrs produced independent α A-crystallin oligomers, 11 $\pm$ 3 nm in diameter (Figs. 6A and B, large arrows). Similar to the observed GP α A-crystallin oligomers (Figs. 5A and B), HU α A-crystallin oligomers were well-defined and homogeneous in distribution with certain images revealing an apparent opening to the interior of the structure (Figs. 6B, small arrows and inset), as has been reported previously for recombinant HU α B-crystallin (Peschek et al., 2009). Based on HPLC analysis (Fig. 2A), the average HU α A-crystallin oligomer would consist of 24 subunits with a MW of 486 kDa. When HU α A-crystallin was incubated with α A^{66–80} peptide for 24 hrs, the presence of linear chains of oligomers, presumably bound together by peptides, was observed, along with small clusters of oligomer aggregates (Fig. 6C), but without formation of fibrils as had been seen for GP α A-crystallin plus α A^{66–80} peptide (Fig. 5C). Linear polymers of human α -crystallin have been observed previously in TEM micrographs (Augusteyn et al., 1989). When HU α A-crystallin was incubated with control V72P peptide, or with α A^{66–80} peptide in the presence of DTT, each for 24 hrs, the formation of oligomer chains and well-defined aggregates was substantially diminished (Figs. 6D and E, respectively). To test whether the presence of only a single crystallin – SH group in HU α A-crystallin, instead of two –SH groups, might lead to peptide-induced fibril formation as was seen for GP α A-crystallin (Fig. 5C), mutant HU C142S α A-crystallin was incubated with α A^{66–80} peptide for 24 hrs. TEM analysis indicated that under this condition, the peptide was not able to induce fibril formation with the crystallin (Fig. 6F); the result was similar to that observed for α A^{66–80} peptide plus WT HU α A-crystallin (Fig. 6C).

3.6 TEM analysis of recombinant GP α A^{ins}-crystallin incubated with peptides

Turbidity and supernatant analyses described above (Fig. 4) had indicated that WI protein aggregates form when GP α A^{ins}-crystallin is incubated either alone or with α A^{66–80} peptide for 24 hrs. TEM analysis was conducted to determine whether amorphous aggregates or structured fibrils form under those conditions. Incubation of GP α A^{ins}-crystallin at time 0 showed the presence of independent oligomers with a mean diameter of 8 $\pm$ 1 nm (Fig. 7A). In contrast to the TEM results for GP and HU α A-crystallins at time 0 (Figs. 5A and 6A, respectively), the GP α A^{ins}-crystallin oligomers exhibited imperfect boundaries with variation in size and shape at the same time point (Fig. 7A). Following 24 hrs of incubation, the crystallins lost their oligomeric structure and formed clustered protein aggregates (Fig. 7B), also in contrast to the results for GP and HU α A-crystallins at the same time period (Figs. 5B and 6B, respectively). Surprisingly, when α A^{66–80} peptide was included in the incubation mixture with α A^{ins}-crystallin for 24 hrs, the peptide was seen to induce the formation of highly-structured peptide/oligomer protofibrils and compressed, mature fibrils

up to nearly 400 nm in length (Figs. 7C, 7F and 9). The average thickness of a protofibril was 12 ± 1 nm (14 fibrils were measured in three micrographs of one experiment) and that of a mature fibril 17 ± 1 nm (6 fibrils were measured in two micrographs of one experiment). The darker staining of the mature fibril at the borders, with lighter staining within the interior regions (Fig. 7F), suggests the existence of a tubule. When αA^{66-80} peptide was replaced with V72P peptide for the 24 hr incubation, amorphous aggregates were detected, with no fibrils (Fig. 7D). Inclusion of the disulfide bond reducing agent DTT in the incubation mixture containing αA^{66-80} peptide and GP αA^{ins} -crystallin also prevented formation of the peptide/oligomer fibrils (Fig. 7E). A comparison of the highly-structured, mature peptide/GP αA^{ins} -crystallin oligomer fibril with the more randomly-formed peptide/GP αA -crystallin oligomer fibril is shown in Figs. 7F and 7G, respectively.

Since the fibrillary nature of a protein is associated with its β -sheet content, β -sheet percentage values for the three HU and GP αA -crystallins were determined using I-TASSER analysis (Table 1). The results indicated 34%, 33% and 30% β -sheet for GP αA -, HU αA - and GP αA^{ins} -crystallin, respectively.

The 23 inserted amino acid sequence LMTHMWFVMHQPHAGNPKNNPAK of GP αA^{ins} -crystallin was further analyzed with use of an online Zyggregator (Tartaglia et al., 2008) program which revealed that the HMWVF continuous stretch of hydrophobic acids in the sequence (residues 67–71) possessed a high probability for fibril formation (a Z_{agg} score >2 , where a score >1 indicates a high propensity to form fibrils) (Supplemental Fig. 4). Similar results were obtained with use of AmylPred2 (Tsolis et al., 2013) and FoldAmyloid programs (Garbuzynskiy et al., 2010) (data not shown).

3.7 Thioflavin T (ThT) fluorescence analysis

To further investigate peptide/ αA oligomer amyloid fibril formation, ThT fluorescence was measured after incubating αA^{66-80} and V72P peptides with recombinant αA -crystallins for 0 and 24 hrs (Fig. 8). ThT selectively localizes to β -sheet-rich structures such as those present in amyloid fibrils to produce an increase in fluorescence brightness (Biancalana and Koide, 2010). When αA^{66-80} peptide, which had previously been shown by TEM analysis to form β -amyloid fibrils (Fig. 3B), was incubated alone for 24 hrs, it exhibited a nearly ten-fold higher level of ThT fluorescence than that for V72P peptide ($p < 0.01$) (Fig. 8). Since αA^{66-80} peptide is itself fibrillogenic, the decision was made to analyze the ThT fluorescence data by comparing values for each crystallin plus peptide to the sums of the individual values for crystallin and peptide. The fluorescence produced by incubating αA^{66-80} peptide with bovine γD -crystallin or GP and HU αA -crystallin for 24 hrs was actually lower than that for the sum of the individual values for peptide and protein (Fig. 8). In sharp contrast, the fluorescence produced by incubation of GP αA^{ins} -crystallin with αA^{66-80} peptide for 24 hrs was 1.6x higher than the sum of the individual values ($p < 0.001$). In addition, the mixture of GP αA^{ins} -crystallin plus αA^{66-80} peptide showed a nearly two-fold fluorescence increase from 0 to 24 hrs ($p < 0.01$), further indication of the formation of β -amyloid fibrils with increased incubation time. Surprisingly, incubation of GP αA^{ins} -crystallin with control V72P peptide showed an 82% increase in fluorescence from 0 to 24 hrs ($p < 0.01$). This result was unexpected since the mixture of GP αA^{ins} -crystallin with the

control peptide was found by TEM analysis to produce only amorphous protein aggregates after 24 hrs, without formation of fibrils (Fig. 7D). This result may have been due to possible interaction of the amyloidogenic region of GP αA^{ins} -crystallin (Supplemental Fig. 4) with hydrophobic amino acids present in V72P peptides, exposing β -sheet-rich structures to yield increased ThT fluorescence.

4 Discussion

This study has demonstrated for the first time the generation of β -amyloid fibrils from a full length lens crystallin protein, GP αA^{ins} -crystallin, incubated under physiological conditions of pH and temperature without use of denaturants (Figs. 7C). Earlier investigations required destabilizing conditions of either low pH or high temperature in order to induce α -, β - and γ -crystallin fibril formation (Ghaffari Sharaf et al., 2017; Meehan et al., 2004; Meehan et al., 2007; Papanikolopoulou et al., 2008; Sharma and Ghosh, 2017; Wang et al., 2010). Indeed, few fibrillation studies of any proteins have been conducted at physiological pH (Yang et al., 2015). The induction of GP αA^{ins} -crystallin fibrils was accomplished by incubating the protein with αA^{66-80} peptide, which forms naturally in the aging lens nucleus and binds to αA -crystallin (Kannan et al., 2013; Raju et al., 2015; Santhoshkumar et al., 2008; Su et al., 2010). The lengths of the fibrils of up to 400 nm (Fig. 7C) are typical of amyloid fibril lengths (Gillam and MacPhee, 2013), and the fibrillation process appeared to be highly organized with classic elongation as is characteristic of amyloid fibril formation (Rambaran and Serpell, 2008). To the best of our knowledge, only one other study has reported fibrillation of αA -crystallin, and that was achieved by incubating HU αA -crystallin under denaturing conditions at 60°C (Meehan et al., 2007).

This study also revealed the tendency of αA^{66-80} peptides to first form independent peptide fibrils in the presence of GP αA -crystallin, preceding a random binding of crystallin oligomers to the fibrils, presumably by hydrophobic bonds, to produce 200–700 nm long peptide/oligomer fibrils (Figs. 5C; Supplemental Fig. 2). It might be argued that GP αA -crystallin oligomers became bound to αA^{66-80} peptide fibrils in a chaperone-like manner in an attempt to prevent further elongation of the fibrils. A similar capability of fibril growth inhibition has been reported for HU αB -crystallin with amyloid- β and αA -synuclein fibrils (Narayan et al., 2012; Raman et al., 2005; Shamma et al., 2011; Stege et al., 1999; Waudby et al., 2010; Wilhelmus et al., 2006). αB -crystallin was shown to bind to the entire length and ends of amyloid- β peptide fibrils as an effective method of inhibiting fibril proliferation (Shamma et al., 2011). Results of a previous study demonstrated the toxic ability of certain fibril-forming peptides to aggregate with each other, as well as with other cellular proteins (Olzscha et al., 2011). The fact that independent αA^{66-80} peptide fibril formation did not take place when the peptide was incubated with either HU αA - or GP αA^{ins} -crystallins may be related to the relative affinities of the peptide for the different crystallins. αA^{66-80} peptide was clearly able to bind to HU αA -crystallin to form amorphous aggregates as evidenced by turbidity and TEM analyses (Figs. 4A and 6C, respectively). Although the binding sites of this peptide to αA -crystallin are not known, the peptide has been reported to attach to αB -crystallin at the C-terminal region (residues 164–174), as well as at and near the chaperone site (residues 73–92) (Kannan et al., 2013). These sites may be more or less exposed in different αA -crystallins.

It is not entirely clear why αA^{66-80} peptide was able to form peptide/oligomer fibrils with GP αA -crystallin (Fig. 5C), but not with the HU protein (Fig. 6C), despite the fact that both crystallins are comprised of 173 amino acids with only eight residue differences (Fig. 1). Mutant GP S142C αA -crystallin, with two –SH groups instead of one (matching the HU protein), lost its ability to form fibrils with the peptide (Fig. 5I). However, mutant HU C142S αA -crystallin, with one –SH crystallin instead of two (matching the GP protein), failed to gain the ability to form peptide/oligomer fibrils (Fig. 6F). Two other GP αA -crystallin mutants, A13T and G153T, were also unable to form fibrils with the peptide (Supplementary Fig. 3). It is possible that the entire 173 amino acid structure of GP αA -crystallin may be required for fibril formation with αA^{66-80} peptide.

Results for turbidity and supernatant analyses (Figs. 4A and B, respectively), and ThT fluorescence analysis (Fig. 8), matched the observed TEM data (Figs. 5C, 6C and 7C). These techniques have been employed previously to investigate αA - and αB -crystallin amyloid fibril formation (Meehan et al., 2007). The combination of αA^{66-80} peptide incubation with GP αA -, HU αA - or GP αA^{ins} -crystallin for 24 hrs each produced increases in turbidity, indicative of either aggregate or fibril formation (Fig. 4A). Supernatant analysis at the 24 hr time point revealed either insoluble aggregate or fibril formation for GP αA - and αA^{ins} -crystallins, but only soluble aggregation of HU αA -crystallin without precipitation (Fig. 4B), all of which was supported by subsequent TEM analysis. ThT fluorescence analysis (Fig. 8) also confirmed the TEM micrograph results indicating αA^{66-80} peptide/crystallin amyloid fibril formation for GP αA^{ins} -crystallin, but not for γD -crystallin and GP and HU αA -crystallins.

The mechanism involved in the compression of 12 nm thick GP αA^{ins} -crystallin protofibrils into 17 nm thick mature fibrils (Fig. 7C) is unclear; however, hydrophobic forces sufficient to pull the peptide/oligomer structures together were presumably involved. It was unexpected to learn by means of I-TASSER analysis that the β -sheet content of GP αA^{ins} -crystallin was actually lower than that for both HU and GP αA -crystallin (Table 1). Presumably, the high propensity for this crystallin to form fibrils with αA^{66-80} peptide is associated to a large extent with the hydrophobic HMWV sequence present in the 23 amino acid insert, which yielded a Z_{agg} score >2 (Supplemental Fig. 4). It can be speculated that the trigger that resulted in the unique formation of αA^{66-80} peptide/GP αA^{ins} -crystallin oligomer fibrils may have been hydrophobic binding of peptide to the HMWV sequence present in the insert.

The disulfide bond reducing agent DTT was found to prevent αA^{66-80} peptide-induced formation of both peptide/GP αA -crystallin fibrils (Fig. 5H) and peptide/ αA^{ins} -crystallin fibrils (Fig. 7E); however, the related mechanisms, and whether disulfide reduction may or may not actually be involved, are not completely clear at this time. Considerable evidence exists to support a role for intermolecular disulfide in the formation and stabilization of certain amyloid fibrils (Fei and Perrett, 2009; Herrmann and Caughey, 1998; Lee and Eisenberg, 2003; Ohhashi et al., 2004; Welker et al., 2001). However, αB -crystallin, which is completely devoid of –SH groups, is also capable of forming fibrils under denaturing conditions (Meehan et al., 2004; Meehan et al., 2007). A possible key factor in the current study is that αA^{66-80} peptide is known to generate H_2O_2 when incubated *in vitro* (Raju et

al., 2017), and thus this peptide would presumably be able to indirectly oxidize crystallin –SH groups during the incubations. Oxidation of HU α A-crystallin with its two –SH groups would have produced intramolecular, and not intermolecular, disulfide as has been documented to occur in HU nuclear cataracts (Takemoto, 1996). This difference may explain why HU α A-crystallin did not form fibrils with α A^{66–80} peptide (Fig. 6C). Since both GP α A- and α A^{ins}-crystallins contain only a single –SH group per subunit, an oxidative environment would produce disulfide-crosslinked oligomer dimers for these two crystallins. It can be speculated that the stacking of GP α A^{ins}-crystallin oligomer dimers one on top of the other in the observed protofibrils (Fig. 9) may have occurred via disulfide-crosslinking of single –SH groups present in the crystallin subunits (disulfide- crosslinked dimers of GP α A^{ins}-crystallin, but not of HU α A-crystallin, can be seen in the SDS-PAGE gels of Supplemental Fig. 5). Similar disulfide-crosslinked oligomer dimers have been observed in acetylcholine receptors from southern ocean electric fish (Tierney et al., 2004).

Although DTT did not appear to have any major effects on the fibrillation of individual α A^{66–80} peptides (Fig. 3C), it cannot be ruled out that the agent was able to block fibril formation (Figs. 5H and 7E) via some non-disulfide-reducing mechanism. A variety of small molecules are known to inhibit fibrillation by affecting β -amyloid oligomerization, fibrillation or both (Necula et al., 2007; Porat et al., 2006). Fibrillation of α A^{66–80} peptide itself was inhibited by epigallocatechin-3-gallate (Kumar et al., 2017) and fibrillation of HU γ D-crystallin and bovine α -crystallin under denaturing conditions was blocked by certain dyes (Sharma and Ghosh, 2017), as well as by carnosine (Attanasio et al., 2009), respectively. In addition to reducing protein disulfide, DTT is known to interact with domains of proteins that lack cysteine residues (Alliegro, 2000), and thus the molecule may have been able to interfere with fibrillation via hydrogen bonding to the GP α A- crystallins.

The observed inability of α A^{66–80} peptide to generate fibrils with HU α A-crystallin (Fig. 6C) matches with reports that fibrils are not present in HU maturity-onset cataracts (Harding, 1972; Meehan et al., 2004), although they have been detected in lenses of Alzheimer's and Down syndrome patients (Goldstein et al., 2003; Moncaster et al., 2010). However, α A^{66–80} peptide did cause aggregation of HU α A-crystallin (Figs. 4A and 6C), which is a hallmark of HU nuclear cataract (Srivastava et al., 2008), and thus this result is supportive of a role for this peptide in HU nuclear cataract formation. It is of interest that in contrast to HU maturity-onset cataracts, amyloid fibrils have been detected in cataractous lenses of the OXYS strain of rats (Marsili et al., 2004), possibly due in part to the fact that rat α A-crystallin contains just a single –SH group and would thus be able to form intermolecular disulfide under oxidative stress.

In the current study, GP α A^{ins}-crystallin exhibited imperfect oligomeric structures during incubation at time 0 (Fig. 7A), and subsequently lost all multimeric structure, breaking apart to form independent monomers and amorphous aggregates (Figs. 2A and 7B). In contrast, other investigators have reported no substantial differences in morphology, multimerization or stability between recombinant rat α A- and α A^{ins}-crystallins (Smulders et al., 1995). This is surprising since there are only two amino acid sequence differences between rat and GP α A^{ins}-crystallin (at residues 85 and 113). Whereas in the previous study, rat α A^{ins}-crystallin was isolated only from the WI fraction of the α A^{ins} cell lysate after unfolding the protein in

6M urea and refolding by dialysis against water or buffer, this technique did not work in our hands for the GP αA^{ins} -crystallin (the protein remained insoluble). Thus, all of the current work was completed only with the 10% GP αA^{ins} -crystallin protein remaining in the WS cell lysate fraction.

αA^{66-80} peptide is known to be present in the old GP lens and to increase in concentration when the animals are treated with HBO (Raju et al., 2015). However, whether the peptide is able to form independent peptide fibrils or induce fibrillation of αA^{ins} -crystallin in the intact GP lens, as was observed *in vitro* in this study (Figs. 5C and 7C, respectively), is not known. Although HBO-treatment of GP's produces an increase in the level of disulfide-crosslinked protein aggregates in the lens nucleus (Simpanya et al., 2005), whether these aggregates might contain αA^{ins} -crystallin amyloid fibrils has not been investigated.

4.1. Conclusions

This is the first time that an α -crystallin, namely GP αA^{ins} -crystallin, has been induced to form amyloid fibrils without the use of denaturants. The triggering factor may have been hydrophobic binding of αA^{66-80} peptide to the highly fibrillogenic HMWV stretch of residues present in the 23 amino acid insertion region of the protein. The existence of a single -SH in the crystallin may have permitted disulfide-crosslinking of αA^{ins} oligomer pairs, which appeared to stack one on top of another in observed peptide/ αA^{ins} oligomer protofibrils. In the presence of normal GP αA -crystallin, αA^{66-80} peptides formed long, independent amyloid fibrils, possibly due to a relatively low affinity of the peptide for the crystallin. The subsequent binding of GP αA -crystallin oligomers to independent peptide fibrils may have been associated with a chaperone-like activity of the crystallin. The observed αA^{66-80} peptide-induced formation of HU αA -crystallin protein aggregates, without fibrillation, supports a role for this age-related lens peptide in the formation of HU nuclear cataract, and is in line with previous reports of an absence of fibrils in HU maturity-onset cataracts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

DEAE diethylaminoethyl

DTT	dithiothreitol
GP	guinea pig
HBO	hyperbaric oxygen
HMW	high molecular weight
HPLC	high pressure liquid chromatography
HU	human
IPTG	isopropyl- β -D-thiogalactopyranoside
mRNA	messenger RNA
MW	molecular weight
NLS	nuclear light scattering
PBS	phosphate buffered saline
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCEP	tris 2-carboxyethyl phosphine
TEM	transmission electron microscopy
ThT	thioflavin T
TRIS	tris(hydroxymethyl) aminomethane
UV	ultraviolet
UVB	ultraviolet B
WI	water insoluble
WS	water soluble
WT	wild type

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Highlights

αA^{66-80} peptide was incubated with lens crystallins under physiological conditions.

Transmission electron microscopy was used to study aggregation and fibrillation.

The peptide first formed independent peptide fibrils with guinea pig αA -crystallin.

The peptide caused aggregation of human αA -crystallin without fibrillation.

The peptide induced mature amyloid fibrillation of guinea pig αA^{ins} -crystallin.

Human	1	MDVTIQHPWFKR	T	LGPFYPSRLFDQFFGEGLEFYDLLPFL	40									
			.											
G.P.	1	MDVTIQHPWFKR	A	LGPFYPSRLFDQFFGEGLEFYDLLPFL	40									
Human	41	SSTISPYRQSLFRTVLDSGISEVR		SDRDKFVIFLDVKHF	80									
G.P.	41	SSTISPYRQSLFRTVLDSGISEVR		SDRDKFVIFLDVKHF	80									
<div style="text-align: center;"> <p>insert LMTHMWFVMHQPHAGNPKNPAK</p></div>														
Human	81	SPEDLTVKVQ	D	DFVEIHGKHNERQDDHGYISREFHRRYRL	120									
			:											
G.P.	81	SPEDLTVKVQ	E	DFVEIHGKHNERQDDHGYISREFHRRYRL	120									
Human	121	PSNVDQSALSCSL	S	ADGMLTF	C	GP	I	Q	T	GLDATHA	ERAIP	160		
					.		:	:		.	:			
G.P.	121	PSNVDQSALSCSL	S	ADGMLTF	S	GP	K	V	Q	S	GLDAGH	S	ERAIP	160
Human	161	VSREEKP	T	SAPSS	173									
			:											
G.P.	161	VSREEKP	S	SAPSS	173									

Figure 1: Amino acid sequences of guinea pig (GP) and human (HU) α A-crystallin.

The eight amino acid differences that exist between GP and HU α A crystallin are shown in red. ":" indicates amino acid residues with similar properties. "." indicates amino acid residues with only weakly similar properties. Residues highlighted in yellow indicate the 15 amino acids comprising the α A⁶⁶⁻⁸⁰ peptide. The 23 amino acids inserted between residues 63 and 64 for GP α A^{ins}-crystallin are also shown.

Figure 2: HPLC and SDS-PAGE analysis of purified recombinant guinea pig (GP) and human (HU) α A-crystallin.

A. Recombinant GP and HU α A-crystallins were analyzed independently by HPLC after applying 25 μ g protein (in 25 μ l PBS) each to a BioSEP 4000 size exclusion column in PBS, pH 7.4, at a flow rate of 1 ml/min. Molecular weight (MW) standards (kDa): a. 2,000; b. 660; c. 443; d. 200; e. 66; f. 29. MWs for GP and HU α A-crystallin were 496 and 486 kDa, respectively (25 and 24 subunits per oligomer). Note that GP α A^{ins}-crystallin eluted mainly as monomers with no oligomeric structure, with some higher MW species. **B.** SDS-PAGE

analysis was conducted after applying 10 µg of each of the five recombinant αA-crystallins to the gel. Lane 1: Molecular weight markers. Lane 2: GP αA-crystallin. Lane 3: mutant GP S142C αA-crystallin. Lane 4: HU αA-crystallin. Lane 5: mutant HU C142S αA-crystallin. Lane 6: GP αA^{ins}-crystallin. The results are representative of three separate analyses.

Figure 3: Transmission electron microscopy (TEM) micrographs of αA^{66-80} and V72P peptides. **A.** i-TASSER images of αA^{66-80} and V72P peptides. Each peptide at 0.05 mg/ml was incubated separately in PBS (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4) at 37°C for 24 hrs and analyzed by TEM. **B.** αA^{66-80} peptide without DTT. **C.** αA^{66-80} peptide with 50 mM DTT. **D.** V72P peptide without DTT. Note the formation of amyloid fibrils in (B) and (C), but not (D). The results are representative of two separate analyses.

Figure 4: Turbidity and supernatant analysis following incubation of α A⁶⁶⁻⁸⁰ and V72P peptides with α A- and γ D-crystallin.

Turbidity analysis at a wavelength of 400 nm (A) was conducted after incubation of α A⁶⁶⁻⁸⁰ and V72P peptides (0.05 mg/ml) with α A- and bovine γ D-crystallin (0.25 mg/ml) for 0 hrs (solid bars) and 24 hrs (open bars). Incubation was conducted in PBS at 37° C. Following turbidity analysis, the samples were centrifuged and the concentration of protein in the supernatant was measured as shown in (B). GP: guinea pig; HU: human; *: $p < 0.05$ compared to the protein by itself. **: $p < 0.01$ compared to the protein by itself. Error bars

represent S.D. for three independent biological repeats. The lack of a visible error bar indicates that the error was too small to be seen.

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Figure 5: Transmission electron microscopy (TEM) micrographs of recombinant guinea pig (GP) α A-crystallin and bovine γ D-crystallin incubated under various conditions.

GP α A-crystallin (0.25 mg/ml) was incubated with and without α A⁶⁶⁻⁸⁰ or V72P peptide (0.05 mg/ml) in PBS at 37° C and analyzed by TEM. **A.** GP α A-crystallin alone, 0 hrs. **B.** GP α A-crystallin alone, 24 hrs. Arrows: GP α A-crystallin oligomers. **C.** GP α A-crystallin plus α A⁶⁶⁻⁸⁰ peptide, 24 hrs. Arrowhead (#1): an apparent independent α A⁶⁶⁻⁸⁰ peptide fibril. Large arrows without tails: GP α A-crystallin oligomers. Arrows with tails (#'s 2, 3 and 4): apparent peptide/ α A oligomer fibrils of various thicknesses. Small arrows without tails: Possible disulfide-crosslinked oligomer dimers. The area highlighted within the box is shown again in Fig. 7G. **D.** Scheme for possible α A⁶⁶⁻⁸⁰ peptide-induced formation of fibrils with GP α A-crystallin as observed in Fig. 5C. A thin α A⁶⁶⁻⁸⁰ peptide amyloid fibril forms first (Stage 1). GP α A-crystallin oligomers bind to the fibril (Stages 2 and 3) to eventually form a thick (>25 nm) peptide/ α A-crystallin oligomer fibril (Stage 4). Bound disulfide-crosslinked oligomer dimers may be present on the fibril. **E.** GP α A-crystallin

plus V72P peptide, 24 hrs. **F** and **G**. Bovine γ D-crystallin (0.25 mg/ml) was incubated either with (**F**) or without (**G**) α A^{66–80} peptide (0.05 mg/ml) in PBS at 37° C for 24 hrs and analyzed by TEM. Note the absence of peptide-induced crystallin aggregates in **F**. **H**. GP α A-crystallin plus α A^{66–80} peptide and 50 mM DTT, 24 hrs. Arrows: Possible thin independent peptide fibrils. **I**. Mutant GP S142C α A-crystallin plus α A^{66–80} peptide, 24 hrs. The average diameter of oligomers present in Figs. A and B: 10 $\pm$ 3 nm (10 oligomers in each of two micrographs were measured, mean $\pm$ S.D.). Thicknesses along the length of each fibril numbered in Fig. C: #1: 9 $\pm$ 3 nm; #2: 25 $\pm$ 11 nm; #3: 11 $\pm$ 3 nm and #4: 21 $\pm$ 8 nm (means $\pm$ S.D. for 10 measurements of each fibril). A-I are representative of 2–3 biological repeats.

Figure 6: Transmission electron microscopy (TEM) micrographs of recombinant human (HU) α A-crystallin incubated under various conditions.

HU α A-crystallin (0.25 mg/ml) was incubated with and without α A^{66–80} or V72P peptide (0.05 mg/ml) in PBS at 37° C and analyzed by TEM. **A.** HU α A-crystallin alone, 0 hrs. Arrows indicate α A oligomers, 11 $\pm$ 3 nm diameter (10 oligomers in one micrograph were measured, mean $\pm$ S.D.). **B.** HU α A-crystallin alone, 24 hrs. Large arrows indicate α A oligomers, 11 $\pm$ 3 nm diameter (10 oligomers in one micrograph were measured, mean $\pm$ S.D.). Small arrows and inset: α A oligomers revealing an apparent opening to the interior of the structure. **C.** HU α A-crystallin plus α A^{66–80} peptide, 24 hrs. Arrowheads indicate peptide/ α A-crystallin oligomer aggregates. Areas highlighted within the boxes indicate linear peptide/ α A-crystallin oligomer chains. **D.** HU α A-crystallin plus V72P peptide, 24 hrs. Arrows with tails indicate amorphous aggregates. **E.** HU α A-crystallin plus α A^{66–80} peptide and 50 mM DTT, 24 hrs. **F.** Mutant HU C142S α A-crystallin plus α A^{66–80} peptide, 24 hrs. Arrowheads indicate peptide/ α A-crystallin oligomer aggregates. Areas highlighted within the boxes indicate linear peptide/ α A-crystallin oligomer chains. Note the absence of well-defined aggregates and chains in Figs. D and E, in contrast to C and F. The results are representative of 2–3 biological repeats.

Figure 7: Transmission electron microscopy (TEM) micrographs of recombinant guinea pig (GP) αA^{ins} -crystallin incubated under various conditions.

GP αA^{ins} -crystallin (0.25 mg/ml) was incubated with and without αA^{66-80} or V72P peptide (0.05 mg/ml) in PBS at 37° C and analyzed by TEM. **A.** GP αA^{ins} -crystallin alone, 0 hrs. Arrows: crystallin oligomers with a mean diameter of 8 $\pm$ 1 nm (ten oligomers were measured in one micrograph; mean $\pm$ S.D.). **B.** GP αA^{ins} -crystallin alone, 24 hrs. Arrows with tails: amorphous peptide/crystallin aggregates. **C.** GP αA^{ins} -crystallin plus αA^{66-80} peptide, 24 hrs. Note the presence of peptide/ αA^{ins} oligomer protofibrils (arrows, 12 $\pm$ 1 nm diameter, 14 protofibrils in three micrographs of one experiment were measured, mean $\pm$ S.D.) and mature fibrils (arrowhead and box, 17 $\pm$ 1 nm, 6 mature fibrils in two micrographs of one experiment were measured, mean $\pm$ S.D.). An enlargement of the mature peptide/ αA^{ins} oligomer fibril shown in the box of Fig. 7C is present in Fig. 7F. Note the darker staining of the mature fibril at the borders, with lighter staining within the interior regions, suggesting the existence of a tubule. **D.** GP αA^{ins} -crystallin plus V72P peptide, 24 hrs. Arrows with tails: amorphous peptide/crystallin aggregates. **E.** GP αA^{ins} -crystallin plus αA^{66-80} peptide and 50 mM DTT, 24 hrs. Arrows with tails: amorphous peptide/crystallin aggregates. **F.** An enlargement of the mature αA^{66-80} peptide/GP αA^{ins} -crystallin oligomer fibril shown in Fig. 7C. **G.** An enlargement of the αA^{66-80} peptide/GP αA^{ins} -crystallin oligomer fibril shown in Fig. 5C. Note the dramatic difference in the αA^{66-80} peptide-induced fibril structures formed by GP αA^{ins} -crystallin and GP αA -crystallin shown in Figs.

7F and G, respectively. Arrows in Fig. 7G: possible disulfide-crosslinked oligomer pairs. The results are representative of 2–3 biological repeats.

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Figure 8: Thioflavin T (ThT) fluorescence analysis following incubation of α A⁶⁶⁻⁸⁰ and V72P peptides with α A- and γ D-crystallins.

ThT fluorescence was measured at a wavelength of 483 nm (445 nm excitation) after incubation of α A⁶⁶⁻⁸⁰ or V72P peptide (0.05 mg/ml) with α A- or bovine γ D-crystallin (0.25 mg/ml) for 0 and 24 hrs. GP: guinea pig; HU: human. a: $p < 0.01$ to V72P peptide, 24hrs; b: $p < 0.05$ to 0 hrs; c: $p < 0.01$ to 0 hrs; d: $p < 0.001$ to the sum of peptide alone plus GP α A^{ins}-crystallin alone, 24 hrs. Error bars represent S.D. for three biological repeats. The lack of a visible error bar indicates that the error was too small to be seen.

Figure 9: Scheme for possible disulfide-crosslinking of guinea pig (GP) αA^{ins} -crystallin oligomers in the peptide/ αA^{ins} oligomer protofibrils of Fig. 7C. Five pairs of stacked αA^{ins} -crystallin oligomers existing in the protofibril are circled in the micrograph. It is speculated that the oligomers may be held together by a combination of binding with αA^{66-80} peptides and intermolecular disulfide-crosslinking as shown in the diagram.

Table 1

Prediction of secondary structure of HU and GP α A-crystallins using I-TASSER analysis.*

	Random Coil (%)	Helix (%)	β -sheet (%)
GP α A	58	7.5	34
HU α A	58	8.7	33
GP α A ^{ins}	59	10.7	30

* Secondary structures of human (HU) and guinea pig (GP) α A-crystallins were analyzed using I- TASSER (<http://zhanglab.ccmb.med.umich.edu/I-TASSER/>).