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Cataract mutation P20S of α B-crystallin impairs chaperone activity of α A-crystallin and induces apoptosis of human lens epithelial cells

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Abstract

Cataract is a common cause of childhood blindness worldwide. α -crystallin, which is comprised of two homologous subunits, α A- and α B-crystallin, plays a key role in the maintenance of lens transparency. Recently, we have identified a missense mutation in α B-crystallin that changes the proline residue at codon 20 to a serine residue (P20S) in a large Chinese family with autosomal dominant posterior polar congenital cataract. To explore the molecular mechanism by which the P20S mutation causes cataract, we examined the quaternary structure, subunit exchange and chaperone activity of the reconstituted heteroaggregates of α -crystallins containing wild type (WT) α A in combination with either WT α B- or mutant α B-crystallin, respectively. Compared with heteroaggregates of WT- α A and WT- α B, heteroaggregates containing WT- α A and mutant α B showed nearly the same molecular mass, but the subunit-exchange rate and chaperone activity were decreased markedly. In human lens epithelial cells, unlike WT α B-crystallin, the P20S mutant protein showed abnormal nuclear localization, and unusual ability to trigger apoptosis. These results suggest that the changes in the structure and function of the α -crystallin complex and cytotoxicity are vital factors in the pathogenesis of congenital cataract linked to the P20S mutation in the α B-crystallin.

1. Introduction

The inherited cataract accounts for about one-third of congenital cataracts, which are a common cause of childhood blindness worldwide [1,2]. The most common mode of inheritance is autosomal dominant. Recently, multiple genes associated with congenital cataracts have been identified. One major class of genes associated with cataracts are genes encoding lens crystallins. The water-soluble lens crystallins constitute approximately 90% of the total lens proteins, and are divided into α -, β -, and γ -crystallin according to the order of their elution on gel exclusion chromatography. These crystallins are very stable proteins and have an essential role in maintaining lens transparency.

The α -crystallin is the most abundant soluble protein in the lens. It is composed of 30 to 40 copies of α A and α B subunits with a 3:1 ratio. The α A and α B crystallins have structural roles within the lens, but are also members of the small heat shock protein family exhibiting important molecular chaperone activity [3-5]. *In vitro* studies demonstrated that the α -crystallin could prevent aggregation of the β - or γ -crystallins [3] by associating with the β - or γ -crystallins at the onset of denaturation, which prevents further unfolding and precipitation of the β - or γ -crystallins *in vivo*. Such chaperone activity of the α -crystallin may be an important factor in preserving lens transparency and protecting against cataracts under various stress conditions and aging.

Genetic studies have linked mutations in α A- and α B-crystallin genes to autosomal dominant congenital cataract [6-10]. Previous studies have shown that mutations of α B-crystallin cause altered structure of the protein [11-13], which results

in reduced chaperone-like activity to suppress protein aggregation, and may lead to the accelerated lens opacification associated with congenital cataract. However, studies of knock-out mice revealed significant differences between the α A- and α B-crystallins [14,15]. Targeted disruption of the α A-crystallin gene in mice induced the cataract phenotype associated with accumulation of inclusion bodies containing a large amount of α B-crystallin. However, disruption of the α B-crystallin gene did not have any effect on the lens transparency. Compared with the α A-knockout mice showing severely decreased chaperone activity, the α B-knockout lenses had relatively normal chaperone activity. These findings suggest that the α A-crystallin may be more essential for maintaining the lens transparency than the α B-crystallin.

Genetic studies firmly link α B-crystallin mutations to the pathogenesis of cataract, but the underlying molecular mechanism remains to be fully defined. We have recently identified a novel mutation, P20S, in the α B-crystallin gene in a large family with autosomal dominant congenital posterior polar cataract [16]. The P20S mutation is the first dominant cataract mutation in α B-crystallin that is located outside of the phylogenetically conserved ' α -crystallin domain'. In this study, we studied the P20S mutation to explore the potentially novel molecular mechanism for the pathogenesis of cataract linked to the α B-crystallin mutations. The results from the knockout mice suggest that the simple loss of function of the α B-crystallin is unlikely to cause cataract as the α B-crystallin knockout mice did not exhibit the phenotype of cataract or abnormal chaperone activity. Thus, we hypothesized that the P20S mutation in the α B-crystallin gene may play a dominant negative role by interacting

with α A-crystallin and interfering with the functions of α A-crystallin. Here we demonstrated that although the P20S mutation had relatively little effect on the oligomeric size of heteroaggregate of α -crystallins, it significantly inhibited the chaperone-like activity and subunits exchange of the α -crystallin complex. Furthermore, in transfected human lens epithelial cells, unlike wild type α B-crystallin, the P20S mutation led to increased localization of the α B-crystallin to the nucleus and triggered apoptosis.

2. Materials and methods

2.1. Cloning and site-directed mutagenesis

The full-length cDNA for human α A- or α B-crystallin genes was cloned between the *Nco* I and *Xho* I sites in plasmid pET-28a for bacterial overexpression. Overlap-extension site-directed mutagenesis was used to generate a fragment with mutation P20S. The fragment was then digested and subcloned into the pET-28a vector between the *Nco* I and *Xho* I sites. Wild type and mutant P20S α B-crystallin genes were also cloned into the pcDNA3.1 mammalian expression vector with a FLAG-tag. The resulting expression constructs were verified by direct DNA sequence analysis.

2.2. Protein expression and purification

The expression plasmids pET28a-WT- α A, pET28a-WT- α B, and pET28a-P20S- α B were transformed into *Escherichia coli* BL21(DE3) competent cells. Culture, induction of protein expression, lysis of cells and purification of the recombinant

proteins were carried out essentially as described by Sun *et al* [17]. The purity of the wild-type (WT) and mutant proteins was examined by SDS-PAGE under reducing conditions and by staining of the protein gels with Coomassie brilliant blue R250 (Sigma-Aldrich, St. Louis, MO). The concentrations of the purified proteins were determined with a Pierce bicinchoninic acid (BCA) assay (Pierce, Rockford, IL).

2.3. Measurement of the size of crystallin oligomers

The purified crystallin was used to investigate the molecular weight of homoaggregates of WT- α B or mutant P20S- α B and heteroaggregates with α A-crystallin. WT- α A (1 mg/ml) was mixed with purified WT- α B or mutant P20S- α B (1 mg/ml) at a 3:1 ratio to form heteroaggregates. To determine the size of oligomers of different aggregates, we used the static light scattering (SLS) method. All proteins were dissolved in 50 mM Tris-HCl, pH 7.9, and filtered through a 0.2- μ m filter. The molecular weight of heteroaggregates was determined by using gel permeation chromatography coupled to low and high angle laser light scattering, and a differential refractive index detector.

2.4. Assay for chaperone-like activity

The ability of the reconstituted α -crystallin heteroaggregates and α A-crystallin homoaggregate to prevent protein aggregation was determined using several substrates. The extent of aggregation was measured by monitoring the light scattering at 360 nm in a UV-8500 spectrophotometer.

Insulin aggregation assay: The aggregation of insulin (0.4 mg/ml, Sigma) in 0.05 M sodium phosphate buffer containing 0.15 M NaCl (pH 7.2) was initialized by the addition of 25 μ l of 1 M dithiothreitol in the presence of WT- α A-crystallin homoaggregate or α -crystallin heteroaggregates containing WT- α A/ α B or WT- α A/ α B mutant or WT- α A/WT- α B/ mutant α B at a 3:0.5:0.5 ratio, respectively. The aggregation was monitored at 37°C.

CS aggregation assay: CS (citrate synthase, 75 μ g, Roche Molecular Biochemicals) in 1 ml of 40 mM HEPES-NaOH buffer (pH 7.4) was heated at 43 °C for 1 h in the presence of various α -crystallin heteroaggregates or WT- α A-crystallin homoaggregate. The light scattering was measured as described above.

γ D-crystallin aggregation assay: γ D-crystallin (250 μ g) in 0.05 M sodium phosphate buffer containing 0.15 M NaCl (pH 7.4) was heated at 63 °C in the presence of different α -crystallin heteroaggregates or WT- α A-crystallin homoaggregate. The light scattering was recorded as described above.

2.5. Subunit exchange

Fluorescence energy transfer was employed to determine the subunit exchange rate in α -crystallin heteroaggregates containing WT α A/WT α B or WT α A/mutant α B. The heteroaggregates was formed by mixture Alexa 350-labeled WT α A with unlabeled WT α B or unlabeled mutant α B at a 3:1 ratio. The exchange reaction was initiated by mixing an equal volume of 0.4 mg/ml Alexa 488-labeled WT α A with 0.4 mg/ml above heteroaggregates containing WT α B crystallin or α B mutant at 37 °C or

43 °C in buffer A. At time 0, 15, 30, 60, 120, and 240 min, 20 µl of the reaction mixture was withdrawn and diluted 100-fold with buffer A (50 mM sodium phosphate, pH 7.5, containing 100 mM NaCl and 2 mM DTT). The emission spectrum of the aliquots (excitation at 420 nm and emission at 520 nm) was recorded using a JASCO FP-750 spectrofluorometer. Since the subunit exchange in α -crystallin is too fast to be reliably measured above 60 °C, we incubate the different heteroaggregates at 63 °C for half an hour and cool them to 4°C immediately, and then compared the subunit exchange at 37 °C. The rate of exchange was obtained by comparing the fluorescence intensities at 520 nm to 420 nm of the fluorescence spectrum, F520nm/420nm.

2.6. Cell culture and transfection

Human lens epithelial cells with extended life span [18] were grown at 37°C in a 5% CO₂ humidified incubator in modified Eagle's medium supplemented with 10% calf serum. Wild type and mutant P20S α B mammalian expression constructs, pcDNA3.1-WT- α B and pcDNA3.1-P20S- α B, were transfected into the HLE cells by the LipofectamineTM2000 Kit according to the manufacturer's instructions. After 48 h, cells were trypsinized and sub-cultured at the ratio of 1:3, and Geneticin was added at a concentration of 0.5 mg/ml for colony selection. Geneticin-resistant colonies were isolated, and individual colonies were expanded into mass cultures. Each colony, presumably derived from a single cell, was examined by quantitative immunoblot analysis and compared with cells transfected with vector only.

2.7. Immunofluorescence

The cellular distribution of WT or P20S mutant α B-crystallin was examined in HLE cells with stable expression of WT or mutant protein. To visualize the distribution of α B-crystallin, cells were incubated overnight in a 1:1000 dilution of an anti-FLAG antibody (Nova Castra). Since the HLE cells constitutively express α B-crystallin, the FLAG-tag was used to distinguish the exogenous and endogenous α B-crystallin. A FITC-conjugated goat anti-rabbit IgG was used as the secondary antibody. Cells were viewed using a laser confocal microscope (Leica Microsystems, Bensheim, Germany).

2.8. Nuclear staining with Hoechst 33342

HLE cells with stable expression of WT or P20S mutant α B-crystallin (1×10^8 /ml) were stained with Hoechst 33342 (Sigma) dissolved in $1 \times$ PBS at a final concentration of 8 μ g/ml at 37 °C for 30 min, and then fixed in 4% paraformaldehyde for 10 min. The stained nuclei were observed by a laser confocal microscope (Leica Microsystems, Bensheim, Germany). Apoptotic cells were morphologically defined as cells showing aberrant, Hoechst 33342-stained nuclei and chromosomal fragmentation.

2.9. Annexin V/PI assays for apoptosis

When analyzing apoptosis using annexin V and PI, the adherent and floating cells

were collected to be stained with annexin V and PI and then evaluated for apoptosis by flow cytometry according to the manufacturer's protocol (Sigma). In brief, 1×10^6 cells were washed twice with PBS and stained with 5 μ L of annexin V-FITC and 10 μ L of PI in $1 \times$ binding buffer (10mM hydroxyethyl piperazine ethanesulfonic acid(HEPES), pH7.4, 140 mM NaOH, and 2.5mM CaCl_2) for 15 min at room temperature in the dark. The apoptotic cells were determined using a FACScan Cytometer (BD Biosciences).

2.10. Statistical analysis

All values were expressed as the mean \pm SD. Statistical analysis was performed using a Student's t-test by the Statistics Software Package. The results were considered statistically significant if $p < 0.05$.

3. Results

3.1. N-terminal mutation P20S in α B-crystallin does not affect the size of reconstituted crystallin heteroaggregates

The α A-crystallin (173 amino acid residues), WT- α B- and mutant P20S- α B-crystallin (175 amino acid residues) were expressed in *E. coli* BL21(DE3) using the pET-28a expression system. Isopropyl β -D-thiogalactoside induction resulted in the overexpression of polypeptides with an apparent molecular mass of 20 kDa, which corresponds to the molecular mass of the α A and α B crystallin subunits (Fig. 1, lanes

2, 4 and 6). After cell lysis, the water-soluble fraction of the lysate was used to purify each recombinant WT- α A, WT- α B, or P20S- α B crystallin. The purified products appeared as a single band on SDS-PAGE (Fig. 1, lanes 3, 5 and 7).

Previous studies showed that the N-terminal region of α -crystallins controlled the degree of oligomerization [19-21]. Since the proline 20 residue is an evolutionarily conserved residue in the N-terminal region of α B-crystallin, its mutation may affect the quaternary structure of α -crystallins. To test this hypothesis, the molecular mass of reconstituted α -crystallins containing WT- α A/WT- α B, WT- α A/mutant P20S α B (α A/ α B = 3:1), homoaggregates of WT- α B or P20S was determined using the static light scattering method. The average molecular mass was 763 kDa for WT- α A/WT- α B, and 748 kDa for WT- α A/P20S- α B. The size of homoaggregate of P20S- α B was also similar to that of WT- α B (Table 1). These results suggest that the P20S mutation of α B crystallin does not dramatically affect the formation of oligomers of α -crystallins.

3.2. The P20S mutation impairs chaperone activity of reconstituted heteroaggregates at physiologically relevant temperatures, but not at high temperatures

To study whether the P20S α B-crystallin affect the chaperone function of α A-crystallin, we compared the ability of different heteroaggregates to prevent protein aggregation with that of homoaggregates of WT- α A-crystallin using various model systems at different temperatures. At 37 °C, the oligomers containing the WT- α A:WT- α B prevented the aggregation of insulin in the presence of DDT just as WT-

α A alone did. In contrast, under the same condition the oligomers containing P20S- α B mutant was ineffective in suppressing the aggregation of insulin compared to WT- α A alone dose. To assess more closely what may occur in the lenses of patients, the oligomers were formed by combining WT- α A, WT- α B, and P20S- α B in their expected in vivo ratio of 3:0.5:0.5. Although WT- α B added into the heteroaggregates could rescue the chaperone activity disrupted by P20S- α B, the heteroaggregates containing WT- α A, WT- α B, and P20S- α B also was ineffective in suppressing the aggregation of insulin compared to WT- α A alone dose (Fig. 2A). With CS as a target protein, at 43°C, the similar results were found as that of insulin (Fig. 2C). Together, these results suggest that the P20S mutation in the N-terminus of α B-crystallin act by a dominant negative mechanism, which interferes with the chaperone activity of wild-type α A-crystallin at the physiologically relevant temperatures.

Interestingly, at a high temperature of 63 °C the oligomers containing P20S- α B mutant prevented the aggregation of γ D-crystallin as WT- α A alone or oligomers containing wild type α B did (Fig. 2E). The results suggest that the P20S mutation of α B-crystallin interfere with the chaperone activity of wild-type α A-crystallin at the physiologically relevant temperatures, but not at a high temperature.

3.3. N-terminal mutation P20S affects the rate of subunit exchange in heteroaggregates at physiologically relevant temperatures

Previous studies showed that the most important requirement for α -crystallin to act as a chaperone is its dynamic structure and the dynamic exchange of subunits [22-

24]. We found that the temperature affected the chaperone activity of the oligomers containing mutant P20S α B, suggesting that mutant P20S α B might alter the dynamic structure of α -crystallin at different temperatures. We therefore studied the dynamic property of the subunit exchange of the wild type and the mutant α B crystallins. To determine the effect of the temperature on the subunit exchange rate of α -crystallins containing P20S α B, we performed the exchange reaction at three different temperatures by FRET analysis. As the exchange progresses, the fluorescence intensity of donor decreases with a concomitant increase in the acceptor fluorescence upon excitement of the sample with the donor absorption maximum, 332 nm. The ratio of the fluorescence intensity at the emission maximum of the acceptor Alexa 488 (520 nm) to that of the donor Alexa 350 (420 nm) monitored as a function of incubation time would thus reflect the rate of subunit exchange. As shown in Fig. 2B and 2D, the rate of subunit exchange for the heteroaggregates containing WT α A-crystallin and mutant α B-crystallin is significantly lower than that for heteroaggregates with WT α A-crystallin and WT α B-crystallin at 37°C or 43°C. These results suggest that mutation P20S of α B-crystallin may alter the interaction between the crystallin subunits, slow the subunit exchange rate, and may make the heteroaggregate more stable, which allow the heteroaggregate not easily disassociate at the physiologically relevant temperatures.

Surprisingly, at a high, non-physiological temperature, the mutant P20S α B-crystallin did not show any difference with wild type α B-crystallin (Fig. 2F). Since the subunit exchange of α -crystallins is too fast to be reliably measured above 60°C

[25], we incubated the different heteroaggregates at 63°C for 30 min and then compared the subunit exchange at 37°C [25]. The heteroaggregates containing WT α A crystallin and mutant P20S α B crystallin showed the similar subunit exchange rate to that of heteroaggregates comprised of WT α A crystallin and WT α B crystallin (Fig. 2F).

3.5. Mutation P20S affects subcellular distribution of α B-crystallin

The expression construct for wild-type α B crystallin or mutant α B-crystallin, which was FLAG-tagged at the C-terminus, was transfected into HLE cells. The clones that stably expressed either P20S or wild-type α B crystallin were selected for further studies. The protein expression level of mutant P20S or wild-type α B crystallin was 0.5 or 0.7ng/mg soluble protein, respectively, as determined by immunoblot analysis (Fig. 3A). The subcellular distribution of mutant P20S versus wild-type α B crystallin in HLE transfectants was compared using immunofluorescence confocal microscopy. As shown in Fig. 3B, wild-type α B crystallin was localized mainly in the cytoplasm and surrounded the cell nucleus, whereas mutant P20S α B crystallin was present in both the cytoplasm and nucleus (Fig. 3C). These results suggest that mutation P20S affects the cellular localization of α B crystallin.

3.6. Mutation P20S triggered cell death

Previous studies showed that mutations in α A-crystallin (R116C) and α B-

crystallin (R120G) could increase human lens epithelial cell death [26, 27]. To investigate whether mutation P20S of α B-crystallin acquires this detrimental function to cellular homeostasis, we examined the cytotoxic effect of mutation P20S. To detect apoptosis, we examined this possibility by different assays. We utilized Hoechst 33342 staining in order to study the changes in nuclear morphology. The stable expression of P20S α B crystallin increased chromatin condensation and fragmentation (Fig. 4A) compared with cells with stable expression of wild-type α B crystallin or cells transfected with empty vector only, indicating that P20S overexpression promoted apoptotic cell death. We also confirmed these data using FACS analysis. Early apoptotic cells stain with annexin V but are impermeable to PI whereas late apoptotic cells doubly stain for both annexin V and PI. Normal cells stain negatively for both. We showed that P20S increased the percentage of HIE cells in apoptotic state in P20S-expressing cells compared with control cells (Fig. 4B,C). These results demonstrate that the P20S mutation increases apoptosis of human lens epithelial cells.

4. Discussion

Mutations in α -crystallins are associated with the autosomal dominant cataract in humans. We have recently identified a cataract-associated mutation P20S in the α B-crystallin gene in a large Chinese family [16]. The molecular mechanism for the pathogenesis of cataract by this mutation, however, is unknown. The present study provides important cues for the molecular basis for the pathology. Unlike the earlier reported missense mutation R120G of α B-crystallin, mutation P20S lies outside of the

α -crystallin core domain and is located at the N terminus. The Pro20 residue in α B-crystallin is evolutionarily conserved among α B-crystallins [16], which suggests that it plays a critical role in the structure and function of the protein.

Earlier studies have shown that mutant α A- or α B-crystallins causing congenital cataracts could form large aggregates *in vitro* or *in vivo* and these high-molecular aggregates may cause defects of scattering of incident light and cataracts. The published data indicated that the N-terminal region controls the degree of oligomerization of α -crystallins. In the present investigation, we reconstituted heteroaggregates using wild type α A-crystallin together with either wild type or mutant P20S α B crystallin to examine whether mutation P20S could form large aggregates with wild type α A-crystallin. In comparison to reconstituted heterooligomers containing wild type α A and wild type α B-crystallins, nearly the same size of aggregates was generated with wild type α A and mutant P20S α B-crystallins. Thus, it appears that the conserved P20 residue of α B crystallin does not affect the oligomeric status of α -crystallins and that the cataract phenotype associated with this mutation may not be caused by scattering of incident light of huge macromolecules.

Our results on oligomerization of α -crystallins by mutation P20S are consistent with the results from two previous studies. The length of human α A and α B crystallins is similar. However, the N-terminal domain of α A crystallin contains five proline residues, whereas the same region of α B crystallin has nine proline residues. A mutant α B crystallin with the N-terminal domain substituted with that of α A crystallin

still possessed the similar oligomerization property to that of wild type α B crystallin. The number of the proline residues at the N-terminus may not be a critical determining factor for oligomerization of crystallins [28]. Furthermore, deletion of the first 19 residues of the N-terminal domain of α A crystallin, which contains the analogous proline residue (P19) as in the α B crystallin (P20), did not alter the size of crystallin oligomers significantly [29].

The important finding from the present study is that the P20S mutation interfered with the chaperone function of α A-crystallins. The reconstituted α -crystallins containing wild type α A crystallin and mutant P20S α B crystallin or containing wild type α A, wild type α B and P20S α B crystallin in a physiological-related condition both showed lower chaperone-like activity at 37°C or 43°C compared with type α A crystallin alone or a complex with wild type α A crystallin and wild type α B crystallin. However, at a high temperature of 63°C, the oligomers containing mutant P20S α B crystallin could inhibit the aggregation of γ D-crystallin as that containing wild type α B-crystallin did. Disassociation and unmasking of the substrate-binding site by the temperature or other post-translational modifications such as phosphorylation appears to be a mode of regulation of the activities of α - crystallins. Pasta [30] *et al* have found that a conserved SRLFDQFFG sequence motif in the N-terminal region of α B-crystallin located from the 21th residue to 29th residue is one of the critical motifs in structure-function regulation of α B-crystallin. When this motif was truncated, the mutant protein exhibited increased chaperone-like activity and increased rate of subunit exchange compared with the wild type proteins. It is reasonable to speculate

that the mutations in this region may affect the interaction of the sequence motif with other parts of the molecule and modulate the structure and chaperone function of the protein. Pro20 in the N-terminal region of α B-crystallin is next to the SRLFDQFFG motif. We speculate that the P20S mutant may have higher affinity with other subunits in the heteroaggregates, which could result in a more stable heteroaggregate and could not disassociate to release the α A-crystallin subunit at physiologically relevant temperatures. It was suggested that a suboligomeric particle disassociated from a large oligomer may act as the primary substrate-binding species, followed by re-assembly into a larger complex with the substrate. To test this, we have carried out the subunit exchange assay with FRET analysis. Compared with the heteroaggregates containing wild type α B, the heteroaggregates containing P20S α B mutant exhibited a lower subunit exchange rate at 37°C or 43°C. At an elevated temperature of 63°C, the heteroaggregate containing the P20S α B mutant showed almost the same subunit exchange rate as heteroaggregates containing wild type α B. These results suggest that mutation P20S may alter the dynamic structure of heteroaggregates and may not normally expose substrate binding sites at physiologically relevant temperatures, but at elevated temperatures the buried or inaccessible substrate binding sites may be unmasked. The effect of P20S on subunit exchange at physiologically relevant temperatures may lead to compromised chaperone activities of α -crystallins and contribute to the development of cataracts.

Lens epithelial cell death appears to be a common cellular mechanism that mediates stress-induced noncongenital cataractogenesis [31, 32]. Mutations in α A-

crystallin (R116C) and α B-crystallin (R120G) result in increased cell death, which is detrimental to cellular homeostasis. Here we show that the P20S mutation of α B-crystallin also significantly increases the level of apoptosis of lens epithelial cells compared to wild type α B-crystallin. Thus, P20S-mediated apoptosis may be an important mechanism for the development of cataract.

The P20S mutant α B-crystallin distributed to both the nuclear and cytoplasmic compartments, whereas the wild type α B-crystallin was mainly in the cytoplasm. These results are consistent with that of the R49C mutation of α A-crystallin [33], which showed distribution in both nuclei and cytoplasm and caused cell death. The molecular mechanisms for nuclear localization of mutant R49C α A-crystallin and mutant P20S α B-crystallin is unknown. However, the cellular localization of P20S α B-crystallin may be related to phosphorylation. There are three phosphorylation sites at the N-terminus of α B-crystallin, Ser19, Ser45, and Ser59. It is possible that the Pro20Ser mutation may interfere with the phosphorylation of α B-crystallin due to its close location with the Ser19 phosphorylation site, and lead to its nuclear import. The abnormal cellular distribution of mutant α B-crystallin in the nuclei may cause cytotoxicity and apoptosis. Recent studies suggested a possible nuclear role for α B-crystallin, which might involve in nuclear splicing events [34-36]. The abnormal accumulation of mutant α B-crystallin in the nuclei could disturb the normal nuclear role for α B-crystallin and result in the apoptosis.

We conclude that P20S of α B-crystallin is a functional mutation that interferes with the function of α A-crystallin, leading to reduced chaperone activity of α A-crystallin

oligomers. Furthermore, the mutation results in increased nuclear import of α B-crystallin and increased apoptosis of lens epithelial cells. All these mechanisms may contribute to the development of cataract in the affected individuals.

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TABLE 1. Determination of the molecular mass of various crystallin oligomers (aggregates).

Type of crystallin	Molecular weight of aggregates*
WT- α B	558 kDa
P20S- α B	539 kDa
WT- α A + WT- α B (3:1 ratio)	763 kDa
WT- α A + P20S- α B (3:1 ratio)	748 kDa

WT, wild type.

*The size of the oligomer or aggregate was measured by the static light scattering method with both 15° and 90° detectors.

Figure legends

Fig. 1. Expression and purification of recombinant human α A-crystallin, wild type (WT) α B-crystallin, and mutant P20S α B-crystallin in *E.coli*. SDS-PAGE was used to analyze the expression and purification of human α A-, WT and P20S α B-crystallins. Lane 1, molecular weight standards; Lanes 2, 4, and 6, crude cell lysates of bacterial cultures transformed with pET28a-wt α A, pET28a-wt α B, and pET28a-P20S α B after induction with 0.1 mM isopropyl- β -D-thiogalactopyranoside, respectively; Lanes 3, 5, and 7, purified WT α A-crystallin, WT α B-crystallin, and mutant P20S α B-crystallin, respectively.

Fig. 2. Chaperone activities of different reconstituted α -crystallin heteroaggregates and WT- α A crystallin homoaggregates, and comparison of subunit exchange rates between heteroaggregates comprised of WT- α A:WT- α B and WT- α A:P20S α B by FRET analysis. The chaperone activity assays were performed with three target proteins and at three different temperatures (A, C, and E). Protein aggregation was determined by monitoring light scattering at 360 nm. Chaperone activity is represented as the percentage protection provided by the wild type and mutant proteins. A, aggregation of insulin B chain by reduction with DTT in the presence of different chaperon/insulin ratios at 37 °C. C, aggregation of CS in the presence of different chaperon/CS ratios at 43 °C. E, aggregation of human recombinant γ D-crystallin in the presence of different chaperon / γ D-crystallin ratios at 63 °C. A mixture (0.4 mg/ml each) of the heteroaggregates containing Alexa 350-labeled WT -

α A (donor) and unlabeled various α B at a 3:1 ratio with Alexa 488-labeled WT - α A (acceptor) was incubated at 37 °C, 43 °C or 63 °C which was examined at 37 °C. a 20 μ l sample was withdrawn at the indicated time intervals and diluted to 0.4 ml with phosphate buffer, and the fluorescence spectrum was recorded with the excitation wavelength of 332 nm. Comparison of subunit exchange in WT - α A : WT - α B and in WT - α A: P20S α B at 37 °C(B) , 43 °C(D), or 63 °C(F) as a function of time. F520 nm/F420 nm represents the ratio of fluorescence intensities at 520 to 420 nm of the fluorescence spectrum.

Fig. 3. Stable expression and cellular distribution of different α B-crystallins in the HLE cells. (A) Western blot analysis demonstrating expression of wild type α B-crystallin (lane 2) and mutant P20S α B-crystallin (lane 3) in HLE cells. Lane 1, transfection with vector only as negative control. (B) Immunofluorescence confocal microscopic images of HLE cells transfected with the expression plasmid encoding wild-type α B-crystallin. Distribution of wild-type α B-crystallin is in the cytoplasm. (C) Immunofluorescence confocal microscopic images of HLE cells transfected with the expression plasmid encoding mutant P20S α B-crystallin. Note the localization of the P20S mutant α B-crystallin in both the cytoplasm and nucleus.

Fig. 4. Effect of mutant P20S α B-crystallin expression on the apoptosis of HLE cells.

(A) The cells were stained with the Hoechst 33342 fluorescence dye, and finally observed under a fluorescent microscope (Magnification: $\times 400$). Data are

representative of at least two independent experiments with similar results. (B) Representative flow cytometry Results. Apoptosis was further measured by flow cytometric analysis of annexin V–FITC and PI staining. Percentages of apoptotic cells (annexin V positive, PI negative and annexin V/PI double positive) were calculated (C). Data are mean \pm SD of three independent experiments. Significant differences from control * $p < 0.05$, ** $p < 0.01$.

Fig1

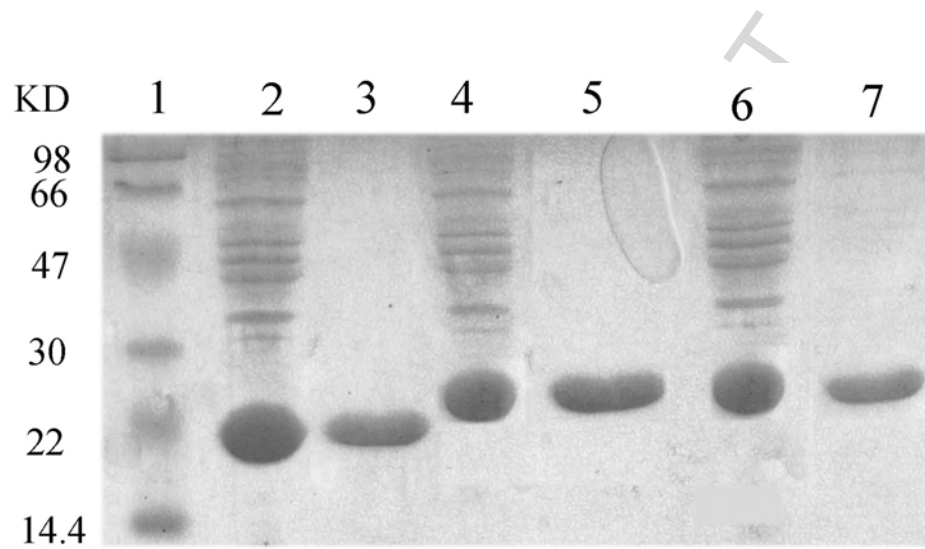


Fig2

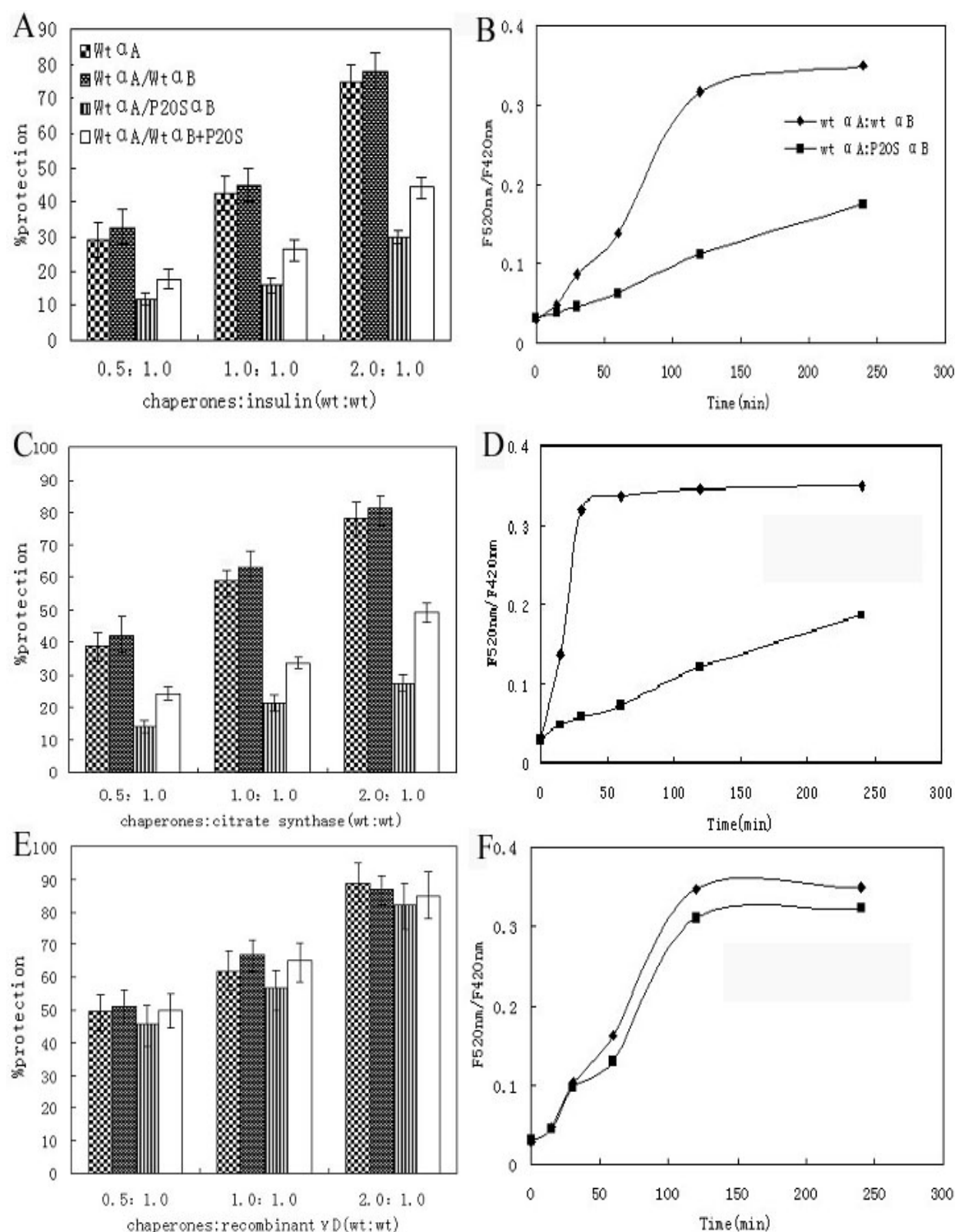


Fig3

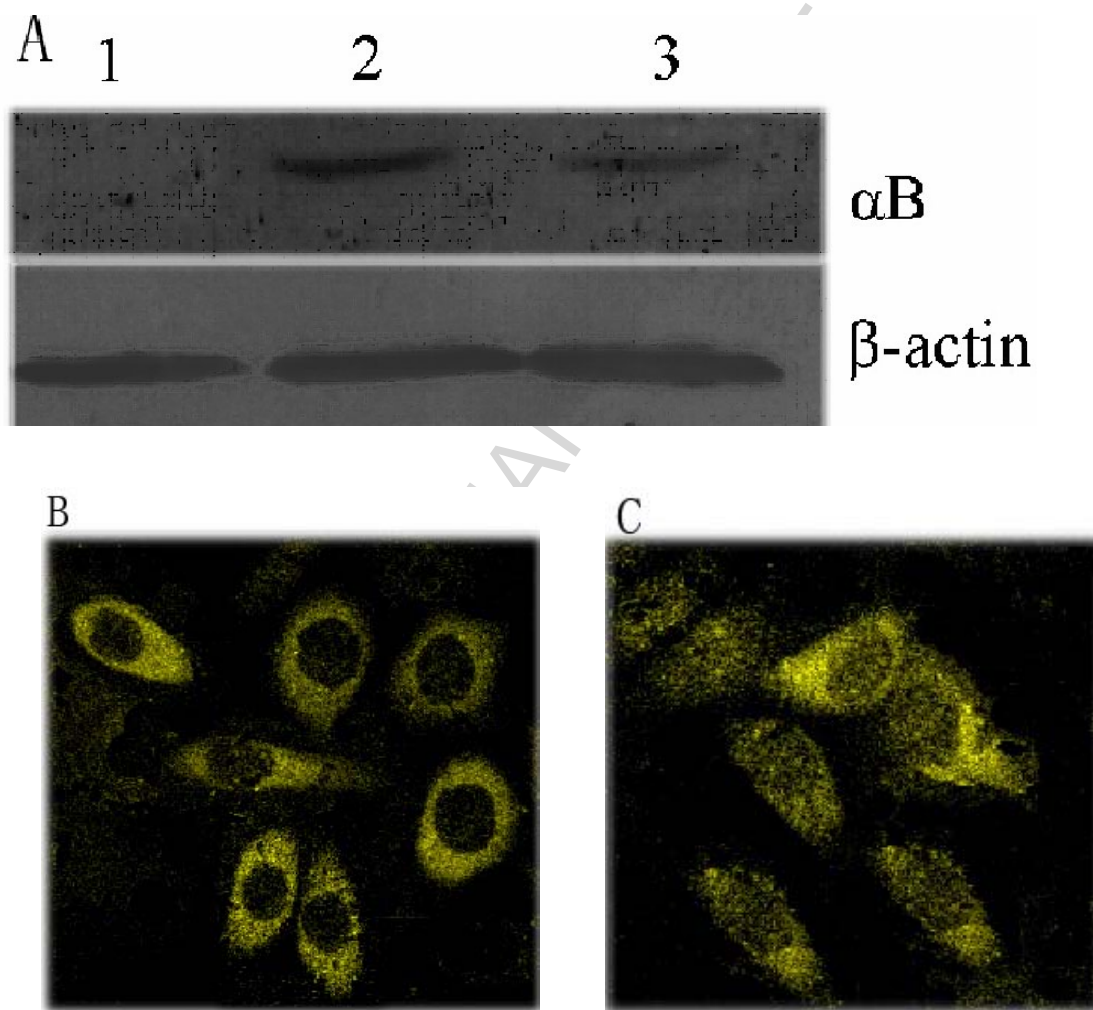


Fig4