Cell Penetration Peptides for Enhanced Entry of α B-Crystallin into Lens Cells

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PURPOSE. The prevalence of cataract increases with age. Conversely, the abundance of native α -crystallin diminishes with age and cataract development. We hypothesize replenishing lens α -crystallin may delay or prevent cataract. Herein we investigated the ability of cell penetration peptides (CPP) to enhance entry of α -crystallins into lens-derived cells.

METHODS. Recombinant α B-crystallins were modified by the addition of CPPs. Candidate CPP were designed with reference to the HSV-1 glycoprotein C gene (gC) or the HIV-1 TAT peptide. α B-crystallins produced by fusing gC or TAT were over-expressed in *E. coli*. Purified proteins were subjected to size exclusion chromatography (SEC) to characterize oligomeric complexes (OC). Chaperone-like activity (CLA) was evaluated by measuring the ability of α -crystallins to suppress chemically-induced protein aggregation. To evaluate protein uptake, labeled α -crystallins were incubated with HLE B3 cells and monitored by fluorescence microscopy for 48 hours.

RESULTS. We examined the effects of the addition of CPP on the structure, CLA, and cell transduction properties of α B-crystallins. C-terminal CPP fused crystallins had poor solubility. In contrast, N-terminal tagged α B-crystallins were soluble. These modified α B-crystallins formed OC that were larger than wild-type based on SEC. Wild-type and gC tagged α B-crystallin displayed robust CLA. Subunit exchange was observed when gC-fused α B-crystallin was mixed with α A. In contrast to wild-type, modified α -crystallins accumulated in HLE B3 cells.

CONCLUSIONS. Addition of CPP improves the uptake of α B-crystallins into HLE B3 cells. No undesirable changes to the chaperone-like abilities of α -crystallins were observed in α B-crystallin modified by the addition of the gC-derived CPP. (*Invest Ophthalmol Vis Sci.* 2013;54:2-8) DOI:10.1167/iov.12-10947

Cataract, characterized by the loss of transparency of the eye lens, is the leading cause of blindness worldwide.¹ With aging, proteins in the lens are damaged by oxidation, deamination, or truncation resulting in unstable proteins that have a higher propensity to aggregate, and thus promote

cataract formation.² The lens is composed primarily of crystallin proteins α , β , and γ .³ The most abundant of these, α -crystallin, is found in both the soluble and insoluble fractions of the lens and can account for as much as 50% of the total protein.^{3,4} α -crystallin is a member of the small heat shock protein family and has chaperone-like activity that suppresses aggregation of other lenticular proteins, thus delaying the onset of cataract.⁵⁻⁸

Alpha crystallins are found in the lens in two forms, α A- and α B-, which form hetero-oligomeric complexes containing 30 to 40 subunits in a ratio of approximately 3:1 (A:B).⁹ Each α -crystallin subunit is ~20 kDa; thus, α -crystallin complexes are approximately 600 to 800 kDa.³ These complexes have been reported to be highly dynamic with subunits exchanging in and out of oligomeric complexes.^{10,11} Mice deficient in α A display an early onset cataract phenotype. In contrast, α B-deficient mice have minimal changes in lens morphology.¹² These differences suggest that while both subunits form oligomeric complexes, the two subunits are not functionally equivalent and their requirement in prevention of cataract may be different.

The depletion of low molecular weight α -crystallin complexes in lens cells has been associated with the onset of cataract.¹³ Because the bulk of lens protein synthesis takes place only in epithelial and outer cortical lens cells, the lens is not well-suited as a target for gene-based therapy aimed at protein replacement. To bypass this limitation, we seek to develop the means to provide therapeutic proteins to lens cells. A challenge inherent to this strategy is how to move therapeutic proteins across the cell membrane. Several reports have identified peptides that, when added to proteins, allow for transduction of proteins across the membrane.¹⁴⁻¹⁸ These peptides, referred to as cell penetration peptides (CPP), interact with membrane surface glycosaminoglycans to mediate their uptake.^{19,20} While the exact mechanism of uptake has not been resolved, previous work has suggested that several different pathways could be involved in CPP uptake.²¹

Since the lens is a highly specialized tissue with surface glycosaminoglycans, peptides with known interactions with lenticular glycosaminoglycans may assist in protein uptake into the lens. Herpes simplex virus type 1 (HSV-1) has previously been shown to infect murine lens and is known to interact with cell surface heparin sulfate.^{22,23} Furthermore, a specific peptide within HSV-1 glycoprotein C (gC) has been shown to interact with heparin sulfate, an early step involved in virus attachment and subsequent membrane fusion.^{24,25} These characteristics of HSV-1 gC suggest its heparin sulfate-binding peptide may also function as a CPP.

Herein, we test the hypothesis that human α B-crystallin can be modified to increase cellular uptake by lens epithelial cells, furthering its therapeutic potential. A recombinant human α B-crystallin was modified to contain either a HSV-1 gC peptide or the conventional human immunodeficiency virus type 1 (HIV-1) TAT peptide. Following expression and purification from *Escherichia coli* host cells, recombinant proteins were

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functionally characterized for their ability to retain α -crystallin properties including, formation of oligomeric complexes, to undergo, subunit exchange, and to have the chaperone-like ability (CLA) to suppress protein aggregation. Purified α -crystallins were further assayed for protein uptake by human lens epithelial B3 cells (HLE B3). Our results show that the addition of both CPPs allows for significant uptake of α B-crystallin in cultured cells. However, unlike the addition of the HIV-1 TAT peptide, fusion of the gC peptide to α B-crystallin does not diminish its properties as a chaperone-like protein.

MATERIALS AND METHODS

Reagents

Construction of Recombinant Human α B-Crystallin Containing Fused Tat or gC CPP Cell Transduction Domain.

Primers for either the TAT or gC CPP were designed with restriction endonucleases (*Nco*I, 5' forward primer; New England Biolabs, Inc., Ipswich, MA; *Hind*III 3' reverse primer; New England Biolabs, Inc.) sequences and were obtained from IDT (Coralville, IA). PCR was carried out using these primers together with our previously described α B-crystallin expression construct²⁶ to prepare TAT- α B and gC- α B coding regions with the CPP at either N- or C-terminus of α B. PCR was performed for 30 cycles of 95°C, 50°C, and 72°C for 1 minute each. Reaction products were separated on a 1% agarose operated in Tris-acetate EDTA (TAE) buffer. PCR bands of the expected size were excised from the gel and purified using the quick spin kit (Qiagen, Germantown, MD) into 30 μ L of 50 mM Tris-HCl pH 8.5. Purified PCR DNA along with pET-23d vector DNA were digested with restriction endonucleases (New England Biolabs, Inc.). Restriction fragments were resolved by 1% agarose TAE gel electrophoresis and appropriate fragments were excised. Purified vector DNA and PCR amplified DNA were ligated together with quick ligase kit (New England Biolabs, Inc.) and 2 μ L were transformed into *E. coli* strain (TOP 10; Life Technologies, Carlsbad, CA). Colonies were selected and inserts confirmed by DNA sequencing.

Expression and Purification of α -Crystallin Constructs. Construction of wild-type human α A- and α B-crystallin cDNA expression clones has been previously described.^{26,27} For all expression clones, plasmids were transformed into *E. coli* strain BL 21(DE3) cells (Life Technologies). Seed cultures of 50 mL were started and grown overnight. Protein expression was performed in 4 $\times$ 400 mL cultures of M9CA plus trace metals and 100 μ g/mL ampicillin as described previously.^{26,27} Cultures were grown for 4 hours at 37°C to an OD₆₀₀ = ~0.7. Cultures were induced with IPTG (final concentration of 1 mM) and grown overnight at 37°C. Bacteria were harvested by centrifugation at 5400g for 15 minutes. The resulting pellet was suspended in 100 mL of N-lysis buffer (50 mM Tris 300 mM NaCl and 0.5 mM EDTA pH 7.5) and lysed by three passages in a French press (ThermoFisher, Waltham, MA) at 1500 psi. Lysed cells were centrifuged at 27,000g for 30 minutes at 4°C. The soluble protein fraction was dialyzed overnight against 4 L of 5 mM sodium phosphate pH 7.5 and 0.5 mM DTT for TAT- α B or 50 mM Tris-HCl, 0.5 mM EDTA pH 7.4, and 0.5 mM DTT for other α crystallins (buffer A) in order to remove salts and optimize the buffer for ion exchange chromatography. Dialyzed protein was centrifuged again at 27,000g for 30 minutes at 4°C. The supernatant material was then loaded onto an ion exchange column: hydroxyapatite (HA) for TAT- α B, Macro S for gC- α B, and Macro Q for wild type α A- or α B-crystallins. Following a column wash with either 100 mL of 100 mM sodium phosphate pH 7.5 and 0.5 mM DTT (buffer B: HA column) or 100 mL of buffer A (Macro Q/S columns), proteins were eluted with a 0 to 500 mM NaCl gradient in either buffer B or buffer A, respectively. Based on SDS-PAGE profiling, fractions positive for α -crystallin were pooled and concentrated using an Amicon pressure concentrator fitted with a 25-kDa molecular weight cutoff filter (Millipore, Billerica, MA). Recombinant α -crystallin proteins were further purified by gel-filtration using

a column (Sephacryl S-400 HR; GE Healthcare Life Sciences, Pittsburgh, PA) and eluted with PBS. Fractions enriched for the protein of interest were pooled, concentrated as before, analyzed by 4% to 20% SDS-PAGE to confirm purity, and quantified using the BCA assay (Pierce, Rockford, IL). Purified protein was stored at 4°C, or at -80°C for long-term storage.

High Molecular Weight Complex Formation Determination.

Purified α -crystallins were loaded onto a Superose 6 size exclusion column (SEC) using an AKTA FPLC (GE Healthcare, Waukesha, WI). Proteins were eluted with PBS into 1-mL fractions. The elution profiles of α -crystallins were monitored in-line by absorbance (280 nm) and plotted against size standards including thyroglobulin (~660 kDa) and ovalbumin (~45 kDa).

α -Crystallin Conjugation to AlexaFluor-488. Purified crystallins were conjugated to dye tags (AlexaFluor-488; Life Technologies) according to the manufacturer's protocol. Briefly, protein in PBS was mixed with 100 mM sodium bicarbonate added to the dye (Life Technologies) and incubated at room temperature for 1 hour on a stir plate. Labeled proteins were then dialyzed overnight against PBS at 4°C to remove excess label. Protein concentrations and percent labeling were determined as recommended by the manufacturer.

Measurement of Chaperone-Like Activity. Assays were performed similar to those previously described.^{5,7} Suppression of protein aggregation by α -crystallin constructs was measured at 37°C using DTT-induced lysozyme aggregates as client proteins, as described previously.^{28,29}

This method was selected because assays are conducted at physiological temperature rather than extreme conditions as in thermal aggregation assays reported in other studies. Briefly, 10 μ M lysozyme (EMD Millipore, Philadelphia, PA) was mixed with 2 mM DTT in the presence or absence of 10 μ M WT- α B-, TAT- α B-, gC- α B-crystallin. Final reaction volumes of 1 mL were obtained by the addition of PBS. Reactions were incubated at 37°C for 1 hour in a Cary 1E UV/vis spectrophotometer (Varian Inc., Walnut Creek, CA), and light scattering continuously monitored at 360 nm for up to 60 minutes.

Subunit Exchange Measurements. Wild type α A-crystallin (100 μ M) was incubated with dye-labeled (Life Technologies) gC- α B (10 μ M) for 0 and 8 hours. The amount of subunit exchange was measured by subjecting the mixture to Superose 6 SEC. Elution of protein complexes were monitored inline by absorbance at 280 nm. Selected fractions were subsequently analyzed for fluorescence (ex 494 nm and em 519 nm) using a fluorescence microplate reader (BioTek Instruments, Winooski, VT).

Cellular Uptake of α -Crystallin Proteins. HLE B3 cells were plated at approximately 40% confluence of a 35-mm glass bottom plates and allowed to adhere overnight. Media from cells was removed and replaced with 400 μ L of reduced serum media (Opti-MEM; Life Technologies). A total of 5 μ g of dye-labeled (Life Technologies) WT- α B, TAT- α B, or gC- α B crystallins were added to the culture medium and incubated for 1 hour at 37°C. Afterward, medium used with labeled proteins was aspirated and replaced with standard growth media and incubated for 24 or 48 hours. At indicated time points, media was removed and Hoechst stain in PBS was added to cells and incubated at 37°C for 15 minutes. Cells were then analyzed by confocal microscopy.

Confocal Microscopy. Confocal imaging was performed using a laser scanning confocal microscope (LSM 510 META on Axiovert 200M platform; Carl Zeiss MicroImaging GmbH, Göttingen, Germany) with control software (Zeiss 510, Zen 2009; Carl Zeiss MicroImaging GmbH) and a Plan-Apochromat 63 $\times$ / 1.4 NA objective. Media was removed and the replaced with PBS. Fluorescence from the tagged (Life Technologies) proteins was detected using the 488-nm spectral line from the equipped Argon laser as an excitation source in combination with a 500- to 550-nm-band-pass emission filter. Cell nuclei were counter stained with the cell-permeable dye Hoechst 33342 (Life Technologies) and imaged using a two-photon Ti:Sapphire laser (Chameleon Ultra II; Coherent Inc., Santa Clara, CA) operating at 800 nm in combination with a 390- to 465-nm-band-pass emission filter.

FIGURE 1. Recombinant α B-crystallin with N-terminal cell penetration peptide tags. (A) CPP sequence from gC or TAT fused to the N-terminus of α B-crystallin. (B) SDS-PAGE analysis of purified gC- α B (left) or TAT- α B (right) from *E. coli*. Proteins were purified as described in Materials and Methods. M, molecular weight marker in kDa.

Images were collected and processed in the control software (Carl Zeiss MicroImaging GmbH).

SDS-Page Analysis of AlexaFluor-488-Labeled Protein. Cells observed by confocal microscopy were analyzed for protein labeling using a conversion screen (XcitaBlue; Bio-Rad, Hercules, CA) analysis. After removal of PBS, cells were frozen at -20°C . Cells were then thawed and scraped into 40 μL of 2 $\times$ SDS loading buffer with 2-mercaptoethanol. Lysates were heated at 100°C for 5 minutes and then loaded onto a 4% to 20% SDS-PAGE. After separation by SDS-PAGE, gels were placed on the conversion screen (Bio-Rad) in a Bio-Rad gel documentation box. Gels were excited with the manufacturers SYBR green settings. Resulting gel images were saved and inverted with Photoshop (Adobe Systems, Inc., San Jose, CA).

RESULTS

Expression of Purified Recombinant α B-Crystallins Containing N-Terminal CPP

In the current studies, we report on the properties of a series of α B-crystallin fusion proteins modified by addition of a 12- or 15-amino acid peptide fused to either the N- or C-terminus of the α B-crystallin subunit. The TAT peptide was based on the known CPP of HIV-1 TAT, while the gC peptide was designed with reference to the product of the gC-gene of herpes simplex virus type 1 (HSV-1 gC; Fig. 1A). When either of these peptides was fused at the C-terminus of α B-crystallin, abundant quantities of the corresponding recombinant protein were detected in *E. coli* expression host cells; however, we found that the proteins of interest were insoluble in detergent-free aqueous buffers used to extract proteins from host cells and were therefore unsuitable for functional studies (data not shown). High protein concentrations or protein misfolding in host cells can result in insoluble products. However, reducing the amount of protein synthesized, either by growing the host cells at suboptimal temperatures or for shorter times after IPTG induction failed to produce desirable amounts of soluble recombinant protein. Furthermore, attempts to refold proteins recovered by urea extraction from the insoluble protein fraction were also unsuccessful (data not shown).

In striking contrast to the C-terminal fusion proteins, we found that CPP addition at the N-terminus of α B-crystallin resulted in soluble protein (Fig. 1B). As with wild type α B-crystallin (WT- α B), the modified proteins containing the CPP

FIGURE 2. CPP-tagged α B-crystallin subunits form high molecular weight OC. Purified TAT- α B (black), gC- α B (blue), and WT- α B (green) crystallin were subjected to Superose 6 SEC. Elution of proteins from SEC column was monitored at an absorbance of 280 nm. Standards of Thyroglobulin (669 kDa) and Ovalbumin (44 kDa) are indicated above chromatogram. gC- α B forms slightly larger OC than WT- α B, while TAT- α B forms even larger OC.

fused at the amino-terminus were successfully isolated to a high degree of purity by sequential column chromatography using ion exchange and size exclusion separation methods (Fig. 1B).

α B-Crystallins Containing N-Terminal CPP Retain the Ability to Form Oligomeric Complexes (OC)

We used size exclusion chromatography to determine if TAT- α B and gC- α B could form OC, and to compare the size of these complexes to the complexes formed by WT- α B. This permitted us to determine if addition of the either peptide affected the ability of α B- to form OC characteristic of WT- α B. All three forms of α B-crystallin were compared with size standards (thyroglobulin and ovalbumin) following FPLC using Superose 6 separation media (Fig. 2). The majority of material detected in the elution profile of gC- α B was consistent with an oligomeric complex of approximately 1.2 MDa. In contrast, α B containing the TAT CPP formed aggregates that were so large as to be excluded from the Superose chromatography medium, and was therefore not suitable for size estimation using this method.

gC- α B, but Not TAT- α B, Retains Significant CLA

The mechanism behind CLA of WT- α B has been extensively studied, as have mutations and posttranslational changes that influence the ability of α -crystallins to function as chaperone-like proteins. However, very few studies have been carried out to probe the functional consequences of adding a CPP sequence to the protein. The CLA of TAT- α B and gC- α B was assayed by measuring their ability to prevent DTT-induced lysozyme aggregation at 37°C . As shown in Figure 3, incubation of lysozyme with DTT at 37°C causes aggregation and an increase in light scattering. The addition of WT- α B or gC- α B largely prevented protein aggregation, whereas TAT- α B had no effect (Fig. 3).

gC- α B-Crystallin Forms Hetero-Oligomeric Complexes with Wild-Type α A

Numerous reports have demonstrated that native α -crystallins form OC that change in size and quaternary organization through the exchange of subunits.^{10,30–32} To assess the ability of gC- α B to undergo subunit exchange, we mixed dye-labeled

FIGURE 3. Analysis of chaperone-like activity of CPP tagged α B-crystallins; 10 μ M Lysozyme mixed with 2 mM DTT in the absence (open diamonds) or presence of TAT- α B (filled circles), gC- α B (triangles) or WT- α B (unfilled circles) at 37°C for 1 hour. The amount of lysozyme aggregation is proportional to the increase in light scattering as measured by the change in absorbance at 360 nm. The effect of 10 μ M subunits of TAT- α B, gC- α B, or WT- α B was monitored in all cases. Addition of gC- α B (triangles) and WT- α B (open circles) greatly reduced light scattering, indicating a great reduction in lysozyme aggregation.

(Life Technologies) protein with a 10-fold excess of wild type α A-crystallin. After an incubation period of 8 hours at 37°C, protein mixtures were resolved by chromatography over a Superose 6 size exclusion column. Fractions were subsequently analyzed for dye (Life Technologies) fluorescence. As shown in Figure 4, dye-labeled (Life Technologies) gC- α B eluted as a sharp peak in the early fractions when analyzed immediately after mixing with wild type α A-crystallin (black arrow, Fig. 4A). However, when samples were analyzed 8 hours after mixing, the dye-labeled (Life Technologies) gC- α B eluted in the later fractions, which correspond to smaller OC (gray arrow, Fig. 4A). This shift indicates that some portion of the gC- α B subunit pool has formed hetero-oligomeric complexes dominated by α A-crystallin. In contrast, incubation of gC- α B for 8 hours in the absence of α A did not result in the reorganization of subunits to form OC similar in size to wild type α A-crystallin (data not shown).

CPP-Tagged α B-Crystallins Show Increased Ability to Enter Cultured Cells

To compare the ability of α B-crystallin to penetrate mammalian cells, we monitored the fate of dye-labeled (Life Technologies) α -crystallins (gC- α B, TAT- α B WT- α B) following a 1 hour exposure to HLE-B3 cells in a cell culture setting. Confocal microscopy of cells 24 hours after the initial exposure showed few cells with noticeable fluorescence, indicating poor penetration of WT- α B under these conditions (Fig. 5A). In contrast, virtually all cells incubated with gC- α B or TAT- α B showed bright fluorescence at the 24-hour time point (Figs. 5B, 5C). At 48 hours, the number of cells that had taken up dye-labeled (Life Technologies) WT- α B increased slightly over the 24-hour time point (Fig. 5D). This suggests that the uptake of WT- α B is likely a slower process. When cells treated with gC- α B or TAT- α B were analyzed at 48 hours, they appeared quite similar to the 24-hour time point (Figs. 5E, 5F). These results indicated that the addition of CPP tag to α B-crystallin greatly enhanced protein penetration into HLE-B3 cells.

To rule out the possibility that intracellular fluorescence arose from degraded protein, we examined protein extracts

FIGURE 4. Subunit exchange of gC- α B with α A-crystallin in OCs. Dye-labeled gC- α B (10 μ M) and unlabeled wild type α A-crystallin (100 μ M) were mixed and incubated at 37°C for 0 (dashed) or 8 (gray) hours (A). Resulting OC were then resolved by Superose 6 SEC. Proteins eluted into 1-mL fractions were assayed for fluorescence using excitation at 494 nm and emission at 519 nm (A) as well as inline measurement of absorbance 280 nm (B). After 8 hours incubation in the presence of α A, there was a decrease in gC- α B fluorescence in fractions 7 to 8, which corresponds to the elution point of high molecular weight OC observed on SEC of gC- α B alone (black-filled arrows [A, B]). The loss of fluorescence associated with this form of OC was accompanied by an increase in gC- α B fluorescence at fractions 14 and 15, which corresponds to the OC dominated by α A (gray-filled arrows [A, B]). For comparison, elution profiles of gC- α B and α A alone are shown in black and gray dashed lines (B).

from either WT- α B- or gC- α B-treated cells using SDS-PAGE and in situ gel fluorescence scanning. Conversion screen (Bio-Rad) fluorescent analysis of the subsequent gels indicated that the dye (Life Technologies) label was localized at a position corresponding to $\sim$ 20 kDa, as would be expected for α B-crystallin subunits (Fig. 6). The fluorescence in extracts produced from gC- α B at 24 and 48 hours were all greater than WT- α B. These results indicate that for at least 48 hours, the fluorescence signal observed in cells using confocal microscopy corresponds roughly to the abundance intact proteins of interest (Fig. 5), and that the penetration of α B is improved by addition of the CPP.

DISCUSSION

A major goal of our studies was to find ways to enhance the ability of α B-crystallin to penetrate into living mammalian cells. We reasoned that modification of the primary structure was an attractive strategy, as this approach would likely be amenable to a range of α B- variants we expect to produce. As a framework, we aimed to produce modified α B-subunits that had enhanced ability to enter into mammalian cells, while concomitantly preserving some functional attributes thought to be important to the chaperone-like functions of small heat shock proteins in general, and α A- and α B-crystallins in particular. These functions include the ability to prevent aggregation of proteins, which likely involves a reorganization of the relatively large oligomeric complex (27-32 mer) by a process of subunit exchange.

FIGURE 5. Confocal microscopy of dye-labeled α B-crystallin uptake in HLE B3 cells; 5 μ g of dye-labeled TAT- α B, gC- α B, or WT- α B was combined with 0.5 mL reduced serum media in a 35-mm dish containing HLE B3 cells. Cells were incubated for 1 hour at 37°C and then media were removed and replaced with fresh reduced serum media without crystallins for up to 48 hours. Nuclei were stained with Hoechst (blue) and labeled α B-crystallin proteins were visualized by dye label (green). Cells were visualized with a 2-photon microscope at 63 $\times$.

Previous studies of recombinant and native α -crystallins have identified structural and functional characteristics that are important for chaperone-like activity. These properties include the formation of OC, the ability to exchange subunits within these complexes, and the ability to suppress aggregation of substrate proteins.^{6,10} These properties are believed to be important for maintenance of the lens both by allowing for short order refraction and prevention of protein aggregation. In vitro studies show that large aggregates are formed following α -crystallin binding in a chaperone-like manner to substrate proteins.³³ Indeed, cataract formation may ensue when the bulk of native α -crystallin complexes are depleted in human lens, presumably converted to high molecular weight complexes as a result of action as a lens chaperone.

We hypothesize that replacement of α -crystallin in the adult lens may provide a therapeutic means to delay the onset or progression of cataracts. Native lens fiber cells are incapable of replenishing stores of α -crystallin if they become depleted due to chaperone-like usage over long periods of time. Following terminal differentiation of lens fiber cells, the intracellular machinery responsible for protein synthesis is lost to degradation. Accordingly, differentiated lens cells have no nuclei and therefore are genetically incapable of gene transcription and mRNA-directed protein synthesis. Therefore, a genetic strategy to replace α -crystallin using vectors engineered to deliver translatable RNA would likely be unsuccessful. We favor an alternate hypothesis that delivery of biotherapeutic proteins may offer more opportunities for success in this setting.

One of the critical challenges for deployment of α -crystallin as an anti-cataract agent is the issue of cell penetration. Previous studies have reported on relatively low levels of native α -crystallin penetration into lens tissue,³⁴ and we have verified this in our own studies (Fig. 5). No cell recognition domains have been identified in the α -crystallin structure to facilitate receptor-mediated binding and uptake into cells. We reasoned that cell uptake could be enhanced by adding a cell transduction domain to α -crystallin subunits. However, we reasoned that any structural modifications to improve transduction should not impair the chaperone-like properties of α -crystallin.

Therefore we sought to characterize CLA on α -crystallin fusion proteins containing CPP (gC- α B and TAT- α B). Our findings indicated that like WT- α B, both gC- α B and TAT- α B formed OC. Not surprising, the small increase in molecular weight of gC- α B resulted in slightly larger OC (Fig. 2) although an increase in subunits cannot be ruled out. When CPP tagged α B-crystallins were examined for chaperone-like activity, gC- α B protected substrate protein from denaturation like WT- α B. In contrast TAT- α B did not protect substrate protein from aggregation, and appeared to increase protein aggregation. This phenomenon could possibly be due to a gain of function as reported with α -crystallin mutants.³⁵ Since TAT- α B formed excessively large OCs and had impaired CLA, only gC- α B was tested for subunit exchange analysis. Analysis of mixing gC- α B with α A indicated that the two proteins underwent subunit exchange of individual subunits between OC similar to those reported with WT- α B and α A. These results indicate that the addition of the gC tag has no detrimental effects on α -crystallin properties.

We propose that the peptide from HSV-1 gC, which has also been shown to interact with heparin sulfate,²⁴ would work as a novel CPP and deliver α B-crystallin into lenticular cells. The addition of this peptide to the N-terminus of α B-crystallin when

FIGURE 6. Detection of α B-crystallin degradation in HLE B3 cells. After a 1 hour exposure of HLE B3 cells to dye-labeled WT- α B or gC- α B cells were incubated for 24 or 48 hours, and subsequently lysed and separated by SDS-PAGE and subjected to Coomassie blue analysis. The amount of dye-labeled α B-crystallins was detected by the appearance of bands at $\sim$ 22 kDa.

expressed in *E. coli* resulted in a soluble protein that could be purified, similar to previous reports with wild type α -crystallin or those with N-terminal tags.^{27,36} Furthermore, gC-tagged- α B-crystallin had similar subunit exchange, CLA and OC, as that of WT- α B. In contrast, the HIV-1 TAT protein tag yielded suboptimal results. These findings suggest that a gC tag on α -crystallin may be used to introduce fresh protein to the lens to delay or prevent cataract.

Wen-Su previously reported that the protein expression pattern in HLE-B3 cells is markedly different from cells freshly dissected, noncultured human lens epithelial cells.³⁷ It is possible that the proteome of native lens epithelial cells, together with factors such as organization of cells into intact monolayers, could influence the uptake of proteins such as crystallins. Therefore, extrapolation of our results from HLE-B3 cells to primary human lens epithelial cells must be considered with caution. However, the basis for using these HLE-B3 cells was solely to assess gC tag-mediated uptake of α -crystallin. Glycosaminoglycans on the surface of primary lens cells would be required to support the binding of our gC peptide necessary to improve cellular uptake. It has been reported that HSV-I infects both nucleated lens epithelial and fiber cells, presumably mediated by the interaction of HSV-gC at the cell surface.²² Since primary lenticular tissues have glycosaminoglycans that are important for HSV-I uptake, we hypothesize that such cells would mediate gC- α B uptake.

While adding the HSV-1 gC peptide tag to α -crystallins may increase protein uptake of wild type protein, the amount delivered to cells is not clear. In addition to delivery of α -crystallin to the lens, additional characterization of α -crystallin's ability to protect and prevent protein aggregation is needed. Several reported cell lines expressing aggregation prone protein mutants and animal models with aggregation prone rhodopsin or γ -crystallin mutants could be used to characterize α -crystallin as a therapeutic agent.³⁸⁻⁴⁰ The fact that we have made a gC- α B that can easily be delivered to cells will allow for the characterization of α -crystallin in this setting. These models will also allow for characterization of any effect that the CPP has on in vivo use of α -crystallin. Further studies are needed to explore the efficacy of therapeutic α -crystallin with regard to prevention of protein misfolding, aggregation and cataract.

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