Cell Penetration Peptides for Enhanced Entry of αB-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 αB-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. αB-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 αB-crystallins. C-terminal CPP fused crystallins had poor solubility. In contrast, N-terminal tagged αB-crystallins were soluble. These modified αB-crystallins formed OC that were larger than wild-type based on SEC. Wild-type and gC tagged αB-crystallin displayed robust CLA. Subunit exchange was observed when gC-fused αB-crystallin was mixed with αA. In contrast to wild-type, modified α-crystallins accumulated in HLE B3 cells.

CONCLUSIONS. Addition of CPP improves the uptake of αB-crystallins into HLE B3 cells. No undesirable changes to the chaperone-like abilities of α-crystallins were observed in αB-crystallin modified by the addition of the gC-derived CPP (Invest Ophthal Mol Vis Sci. 2013;54:2–8) DOI:10.1167/iovs.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, αA- and αB-, 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 αA-display an early onset cataract phenotype. In contrast, αB-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 glycaminoglycans 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 glycaminoglycans, peptides with known interactions with lenticular glycaminoglycans 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 αB-crystallin can be modified to increase cellular uptake by lens epithelial cells, furthering its therapeutic potential. A recombinant human αB-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
functionally characterized for their ability to retain z-crystallin properties including, formation of oligomeric complexes, to undergo, subunit exchange, and to have the chaperone-like ability (CLA) to suppress protein aggregation. Purified z-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 zB-crystallin in cultured cells. However, unlike the addition of the HIV-1 TAT peptide, fusion of the gC peptide to zB-crystallin does not diminish its properties as a chaperone-like protein.

**Materials and Methods**

**Reagents**

Construction of Recombinant Human zB-Crystallin Containing Fused Tat or gC CPP Cell Transduction Domain. Primers for either the TAT or gC CPP were designed with restriction endonucleases (Bgl II, 5’ forward primer; New England Biolabs, Inc., Ipswich, MA; HindIII 3’ reverse primer; New England Biolabs, Inc.) sequences and were obtained from IDT (Corvalle, IA). PCR was carried out using these primers together with our previously described zB-crystallin expression construct26 to prepare TAT–zB and gC–zB coding regions with the CPP at either N- or C-terminus of zB. 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 50 μL of 50 mM Tris-HCl pH 8.5. Purified PCR DNA along with pET22d vector DNA were digested with restriction endonucleases (New England Biolabs, Inc.). Restriction fragments were resolved by 1% agarose 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 μL 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 z- and g-Crystallin Constructs. Construction of wild-type human zA- and zB-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. Cultures were induced with IPTG (final concentration of 1 mM) and plus trace metals and 100 μg/mL ampicillin as described previously. Cultures were grown for 4 hours at 37°C to 1.4 NA objective. Media was removed and replaced with 400 μL of reduced serum media (Opti-MEM; Life Technologies). A total of 5 μg of dye-labeled (Life Technologies) WT–zB, TAT–zB, or gC–zB 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×/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 590- to 465-nm-band-pass emission filter.
To assess the ability of E. coli expression host cells; however, we found that CPP addition at the N-terminus of α-crystallin resulted in soluble protein (Fig. 1B). As with wild type αB-crystallin (WT-αB), the modified proteins containing the CPP 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).

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

In the current studies, we report on the properties of a series of αB-crystallin fusion proteins modified by addition of a 12- or 15-amino acid peptide fused to either the N- or C-terminus of the αB-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 αB-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 αB-crystallin resulted in soluble protein (Fig. 1B). As with wild type αB-crystallin (WT-αB), the modified proteins containing the CPP...
(Life Technologies) protein with a 10-fold excess of wild type αA-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-αB eluted as a sharp peak in the early fractions when analyzed immediately after mixing with wild type αA-crystallin (black arrow, Fig. 4A). However, when samples were analyzed 8 hours after mixing, the dye-labeled (Life Technologies) gC-αB eluted in the later fractions, which correspond to smaller OC (gray arrow, Fig. 4A). This shift indicates that some portion of the gC-αB subunit pool has formed hetero-oligomeric complexes dominated by αA-crystallin. In contrast, incubation of gC-αB for 8 hours in the absence of αA did not result in the reorganization of subunits to form OC similar in size to wild type αA-crystallin (data not shown).

**CPP-Tagged βB-Crystallins Show Increased Ability to Enter Cultured Cells**

To compare the ability of βB-crystallin to penetrate mammalian cells, we monitored the fate of dye-labeled (Life Technologies) α-crystallins (gC-αB, TAT-αB WT-αB) 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-αB under these conditions (Fig. 5A). In contrast, virtually all cells incubated with gC-αB or TAT-αB 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-αB increased slightly over the 24-hour time point (Fig. 5D). This suggests that the uptake of WT-αB is likely a slower process. When cells treated with gC-αB or TAT-αB 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 αB-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 from either WT-αB- or gC-αB-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 ~20 kDa, as would be expected for αB-crystallin subunits (Fig. 6). The fluorescence in extracts produced from gC-αB at 24 and 48 hours were all greater than WT-αB. 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 αB is improved by addition of the CPP.

**Discussion**

A major goal of our studies was to find ways to enhance the ability of αB-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 amendable to a range of αB-variants we expect to produce. As a framework, we aimed to produce modified αB-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 αA- and αB-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.
Previous studies of recombinant and native \( \alpha \)-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 \( \alpha \)-crystallin binding in a chaperone-like manner to substrate proteins.\(^3\)\(^5\) Indeed, cataract formation may ensue when the bulk of native \( \alpha \)-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 \( \alpha \)-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 \( \alpha \)-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 \( \alpha \)-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 \( \alpha \)-crystallin as an anti-cataract agent is the issue of cell penetration. Previous studies have reported on relatively low levels of native \( \alpha \)-crystallin penetration into lens tissue,\(^3\)\(^9\) and we have verified this in our own studies (Fig. 5). No cell recognition domains have been identified in the \( \alpha \)-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 \( \alpha \)-crystallin subunits. However, we reasoned that any structural modifications to improve transduction should not impair the chaperone-like properties of \( \alpha \)-crystallin.

Therefore we sought to characterize CLA on \( \alpha \)-crystallin fusion proteins containing CPP (gC-\( \alpha \)-B and TAT-\( \alpha \)-B). Our findings indicated that like WT-\( \alpha \)-B, both gC-\( \alpha \)-B and TAT-\( \alpha \)-B formed OC. Not surprising, the small increase in molecular weight of gC-\( \alpha \)-B resulted in slightly larger OC (Fig. 2) although an increase in subunits cannot be ruled out. When CPP tagged \( \beta \)-crystallins were examined for chaperone-like activity, gC-\( \beta \)-B protected substrate protein from denaturation like WT-\( \beta \)-B. In contrast TAT-\( \beta \)-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 \( \alpha \)-crystallin mutants.\(^5\) Since TAT-\( \beta \)-B formed excessively large OCs and had impaired CLA, only gC-\( \beta \)-B was tested for subunit exchange analysis. Analysis of mixing gC-\( \beta \)-B with \( \alpha \)-\( \alpha \) indicated that the two proteins underwent subunit exchange of individual subunits between OC similar to those reported with WT-\( \beta \)-B and \( \alpha \)-\( \alpha \). These results indicate that the addition of the gC tag has no detrimental effects on \( \alpha \)-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 \( \beta \)-crystallin into lenticular cells. The addition of this peptide to the N-terminus of \( \beta \)-crystallin when
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.\textsuperscript{27,30} Furthermore, gC-tagged-αB-crystallin had similar subunit exchange. CLA and OC, as that of WT-αB. 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.\textsuperscript{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 the gC peptide necessary to improve cellular uptake. It has been reported that HSV-1 infects both nucleated lens epithelial and fiber cells, presumably mediated by the interaction of HSV-gC at the cell surface.\textsuperscript{22} Since primary lenticular tissues have glycosaminoglycans that are important for HSV-1 uptake, we hypothesize that such cells would mediate gC-αB 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.\textsuperscript{38–40} The fact that we have made a gC-αB 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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**References**


