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Alpha-crystallin-mediated protection of lens cells against heat and oxidative stress-induced cell death ☆

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ABSTRACT

In addition to their key role as structural lens proteins, α-crystallins also appear to confer protection against many eye diseases, including cataract, retinitis pigmentosa, and macular degeneration. Exogenous recombinant α-crystallin proteins were examined for their ability to prevent cell death induced by heat or oxidative stress in a human lens epithelial cell line (HLE-B3). Wild type αA- or αB-crystallin (WT-αA and WT-αB) and αA- or αB-crystallins, modified by the addition of a cell penetration peptide (CPP) designed to enhance the uptake of proteins into cells (gC-αB, TAT-αB, gC-αA), were produced by recombinant methods. In vitro chaperone-like assays were used to assay the ability of α-crystallins to protect client proteins from chemical or heat induced aggregation. In vivo viability assays were performed in HLE-B3 to determine whether pre-treatment with α-crystallins reduced death after exposure to oxidative or heat stress. Most of the five recombinant α-crystallin proteins tested conferred some in vitro protection from protein aggregation, with the greatest effect seen with WT-αB and gC-αB. All α-crystallins displayed significant protection to oxidative stress induced cell death, while only the αB-crystallins reduced cell death induced by thermal stress. Our findings indicate that the addition of the gC tag enhanced the protective effect of αB-crystallin against oxidative but not thermally-induced cell death. In conclusion, modifications that increase the uptake of α-crystallin proteins into cells, without destroying their chaperone-like activity and anti-apoptotic functions, create the potential to use these proteins therapeutically.

1. Introduction

α-Crystallins make up the major protein component of the mammalian lens and function as structural and refractive proteins [1,2]. The two forms of α-crystallin (αA-crystallin and αB-crystallin) share 57% sequence homology [3] and have been reported to form multimeric complexes with each other at a ratio of 3:1 (αB-crystallin:αB-crystallin) [4]. More recent studies suggest that αA- and αB-crystallin are found in distinct membrane compartments within cells [5] and may therefore have additional functions in addition to acting as refractive proteins. These additional functions may differ between each isoform, since αA-crystallin is found almost exclusively in the lens while αB-crystallin is found in multiple tissues including the retina, heart, skeletal muscle, glia, kidney, lung, and Schwann cells [6–9].

In vitro studies of α-crystallins indicated that the proteins function as molecular chaperones based on their ability to promote refolding after denaturation and suppress thermally-induced protein aggregation [10,11]. Additionally, transfection of α-crystallin DNA into cultured cells has indicated its ability to promote cellular thermo-resistance and prevent UVA-induced apoptosis in human lens epithelial cells [12–14]. Furthermore, α-crystallin knockout animal models have decreased resistance against oxidative stress [15]. αB-crystallin has been shown to be up-regulated in cells exposed to heat, osmotic, and mechanical stresses, likely preventing damage induced apoptosis [16–18]. In human lens and retina cells under oxidative stress, αB-crystallin protects mitochondrial cytochrome c from oxidation, preventing apoptosis [19].

We hypothesize that the introduction of α-crystallins to the lens may represent an approach to limit cell death, and progression of cataract. Epithelial cells that progress to cortical fiber cells accumulate large amounts of proteins that must maintain structural integrity for many decades to support lens transparency. Over time, these cells lose the capacity to produce new proteins [1]. It has been hypothesized that in response to metabolic and environmental stresses to the lens, (UV light exposure, oxidative stress secondary to metabolic diseases), α-crystallin binds to both unfolded proteins and those involved in apoptosis, including cytochrome c and caspase 3, to prevent cell death [19–22]. Therefore, increased levels of α-crystallin may delay or prevent cataract.

While delivery of recombinant α-crystallins to tissues offers an intriguing approach to prevent protein aggregation diseases, protein uptake into cells at levels sufficient for efficacy is likely to be a challenge. A peptide in the TAT protein of human immunodeficiency virus-1 (HIV-1) was the first cell penetration peptide (CPP) shown to enter cells non-selectively and without a specific receptor [23]. In 1994, the TAT CPP

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was first utilized to increase protein uptake into cells when Fawell et al. chemically cross-linked part of TAT to proteins which resulted in transduction of otherwise impermeable proteins [24]. Similarly, herpes simplex virus type 1 (HSV) encodes for the glycoprotein C (gC) shown to be involved in viral attachment to cells. We have previously shown that the fusion of either TAT or gC CPP to αB-crystallin led to a significant increase in uptake of α-crystallin to lens-derived cells in culture after a one hour incubation [25]. However, the ability of these proteins to function within these cells is currently unknown. The purpose of the present study was to assess the efficacy of α-crystallins introduced in this manner against various types of stress in a cell culture model.

2. Methods

2.1. Cell culture

Human lens epithelial cells (HLE-B3) [26] were grown in DMEM (Sigma, St. Louis, MO) with 20% fetal bovine serum (SAFC Biosciences, Lenexa, KS) and penicillin–streptomycin–amphotericin B (10 units/ml–10 units/ml–0.25 μg/ml (HyClone, Logan, UT)) at 37 °C in the presence of 5% CO_2. Cells were added to 12-well or 96-well plates (Costar, Corning, NY) and allowed to adhere overnight.

2.2. Cloning of recombinant human αA-crystallin fused with gC cell penetration peptide (CPP)

Construction of αA-crystallin with an N-terminal gC peptide was carried out as previously reported for gC-fused αB-crystallin [25]. Briefly, primers for the gC CPP were designed with *Nco*I (5′ forward primer) and *Hin*dIII (3′ reverse primer) sequences and were obtained from IDT (Coralville, IA). PCR was carried out using these primers together with our previously-described αA-crystallin expression construct [27] to prepare gC-αA coding region with the CPP at the N-terminus of αA. PCR was performed for 30 cycles of 95 °C, 50 °C, and 72 °C for 1 min 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) following the manufacturer's protocol. Purified PCR DNA along with pET-23d vector DNA was digested with *Nco*I and *Hin*dIII (New England Biolabs, Ipswich, MA). Restriction fragments were resolved by 1% agarose TAE gel. Purified vector DNA and PCR amplified DNA were ligated using a quick ligase kit (NEB) and transformed into *Escherichia coli* strain TOP 10 (Life Technologies, Carlsbad, CA). Colonies were selected and inserts confirmed by DNA sequencing.

2.3. Production of recombinant α-crystallin proteins

Preparation of expression plasmids encoding wild-type αA-crystallin (WT-αA), wild-type αB-crystallin (WT-αB), gC-tagged αA-crystallin (gC-αA), gC-tagged αB-crystallin (gC-αB), and TAT-tagged αB-crystallin (TAT-αB) have previously been reported [25,27,28]. Expression and purification of recombinant crystallins were carried out as described [28]. Purified proteins were kept at 4 °C if used immediately, or were stored at −80 °C.

2.4. Cellular heat stress assay

HLE-B3 cells grown to ~90% confluence were treated with a single α-crystallin for 1 h at 37 °C. Briefly, 12.5 μg/ml of α-crystallins (or PBS alone as a vehicle control) was added to the culture medium and cells were incubated for 1 h at 37 °C. The culture medium was then replaced with fresh culture media without α-crystallin. After this media exchange, cells were placed at 45 °C for 1 h to induce heat stress. Following the 45 °C heat treatment, cells were returned to 37 °C. Live cells were identified 23 h later using the Live/Dead Viability/Cytotoxicity Assay Kit (Life Technologies) which relies on cytoplasmic esterases to cleave calcein and generate intracellular fluorescence (calcein assay). To measure this activity, 23 h after heat treatment, cells were washed twice with PBS and placed in 120 μL PBS with 5 μM Calcein AM and incubated for 20 min at 25 °C in the dark. Following incubation, viable cells were detected using an excitation at 485 nm and emission at 528 nm with a Synergy 4 Multi-mode Microplate Reader and Gen5 Reader Control and Data Analysis Software (BioTek, Winooski, VT). After subtracting background fluorescence from all samples, untreated (no protein or heat) cells were used to determine 100% viability. To determine the number of cells protected from heat treatment by α-crystallin, the following equation was used:

$$\%\text{viable} = \frac{\%\,\text{viable with}\,\alpha\text{-crystallin} - \text{avg.}\%\,\text{viable without}\,\alpha\text{-crystallin}}{100 - \text{avg.}\%\,\text{viable without}\,\alpha\text{-crystallin}} \times 100.$$

Results are reported as average ± standard deviation of at least three independent experiments. Each experimental condition was studied in triplicate. The p-values were calculated using a two-tailed, Student's t-Test assuming equal variance.

2.5. Cellular oxidative stress assay

Treatment groups were assembled in the same manner as the cellular heat stress assays described above. The only modification from the above assay is after α-crystallin treatment, cells were exposed to 37.5 μM H_2O_2 (Sigma) and incubated at 37 °C for 24 h. Cell viability after this treatment was assessed using the calcein assay as above.

2.6. Chaperone-like activity measurements on reduction-induced aggregation

Assays were performed similar to those previously described [25,29,30]. Briefly, 10 μM lysozyme (EMD Millipore, Philadelphia, PA) was mixed with 1 mM DTT in the presence or absence of 10 μM WT-αA, WT-αB, gC-αB, TAT-αB, or gC-αA. Reactions were monitored for 1 h at 37 °C in a Cary 1E spectrophotometer fitted with a Peltier controlled sample carrier. Samples were continuously monitored for light scattering at 360 nm for 60 min.

2.7. Toxicity assays

To assess the toxicity of α-crystallin preparations, HLE-B3 cells were incubated for 1 h in the presence of 12.5 μg/ml, 37.5 μg/ml, or 125 μg/ml of each of five variants of α-crystallin prepared for this study. After 1 h, media were replaced with α-crystallin-free media and incubated overnight. 24 h post-α-crystallin exposure, cells were harvested and viability was determined using the Live/Dead Viability/Cytotoxicity Assay Kit (Life Technologies) as above. Assays were repeated in triplicate for each protein.

2.8. Chaperone-like activity measurements on heat-induced aggregation

CLA assays with the human aldose reductase AKR1B1 (HAR) heat-induced aggregation assay have been described previously [27,28]. Briefly, 2.5 μM recombinant HAR was mixed with 1 mM DTT in the presence or absence of WT-αA, WT-αB, gC-αB, TAT-αB, or gC-αA (all at 2.5 μM crystallin subunit concentration). Samples were incubated for 30 min at 53 °C in a Cary 1E UV/vis spectrophotometer. Samples were continuously monitored for light scattering at 360 nm for 60 min.

2.9. α-Crystallin conjugation to Alexa-Fluor-350 and Alexa-Fluor 488

α-Crystallins were conjugated with Alexa-Fluor-350 or Alexa-Fluor 488 fluorophores according to the manufacturer's protocol (Life Technologies). Concisely, α-crystallins in PBS were mixed with

100 mM sodium bicarbonate added to Alexa-Fluor-350 or Alexa-Fluor 488 dye and incubated with mixing at 25 °C for 1 h. Alexa-Fluor dye-labeled proteins were then passed over a kit-supplied desalting column to remove unconjugated dye. Protein concentrations and percent labeling were determined as recommended by the manufacturer.

2.10. Microscopy analysis of α-crystallin

HLE-B3 cells were plated onto 35 mm glass bottom culture dishes overnight to allow adherence. Cells with ~90% confluence were treated with 12.5 μg/ml of Alexa-Fluor-350 labeled α-crystallin for 1 h. The α-crystallin-supplemented medium was then replaced with fresh media containing 37.5 μM H_2O_2 and cells incubated for 24 h. Next, cells were harvested and washed twice with PBS followed by incubation in 1 ml of PBS with 5 μM calcein AM solution at 25 °C for 20 min in the dark. After incubation, cells were imaged using a Nikon 90i microscope at 10× magnification. The Alexa-Fluor-350 labeled α-crystallin detected by blue fluorescence was converted to red in the images.

2.11. Immunohistochemistry

Glass coverslips were placed into 12-well plates. HLE-B3 cells were allowed to adhere to coverslips. After cells reached ~95% confluence, 2 μg of Alexa-Fluor 488-labeled gC-αB were added to the well. After 72 h, coverslips were removed from wells and rinsed in PBS. The coverslip was then placed in 100% methanol for 30 s, rinsed with 1× PBS for 30 s, and were then blocked with 2% w/v BSA in PBS for 30 min. After blocking, α-crystallin antibody (a gift from Dr. Usha Andley, Washington University in St. Louis, MO) at 1/300 dilution in 2% BSA was added for 20 min. The coverslip was then washed with PBS, again blocked with 2% BSA for 30 min, then probed with a 1/500 dilution of goat anti-rabbit 594 (Life Technologies) for 20 min. After another rinse with PBS, the coverslip was mounted on a glass slide with DAPI with VectaShield (Vector Laboratories, Burlingame, CA), and imaged using a Nikon microscopy system.

3. Results

3.1. Co-localization of native and recombinant α-crystallin taken up into HLE-B3 cells

Previously we reported the uptake of α-crystallin protein by HLE-B3 cells [25]. However, the localization of exogenous gC-αB was not determined. Using immunohistochemistry to detect α-crystallin in cells, we found that Alexa-Fluor 488-labeled recombinant, exogenous proteins co-localize with the native α-crystallin in perinuclear regions; however, it did not localize with native proteins at leading edges of cells (Fig. 1). Moreover, exogenous α-crystallin formed punctate patterns in ~5% of cells. This suggests that both the gC tag, as well as the cell uptake mechanism may affect the protein's trafficking upon cell entry.

3.2. Recombinant protein toxicity in HLE-B3 cells

A consequence of using recombinant proteins from *E. coli* is the potential for toxic contaminants during purification. As a first step in assessing the use of α-crystallins in a cell-based system, we tested whether the treatment of HLE-B3 cells with recombinant proteins was associated with toxicity. Treatment of HLE-B3 cells over a 10-fold concentration range using five different α-crystallins was generally not associated with any toxic effects (Fig. 2). Among the five protein preparations tested, only WT-αA showed a statistically-significant reduction in cell viability (76.4% ± 12.4%, $p < 0.01$) but even in this case, the reduction was observed only in cells treated at the highest concentration (125 μg/ml). Additional purification of α-crystallins over polymyxin resin to remove endotoxin did not improve cell viability (data not shown). An apparent increase in viability was observed in

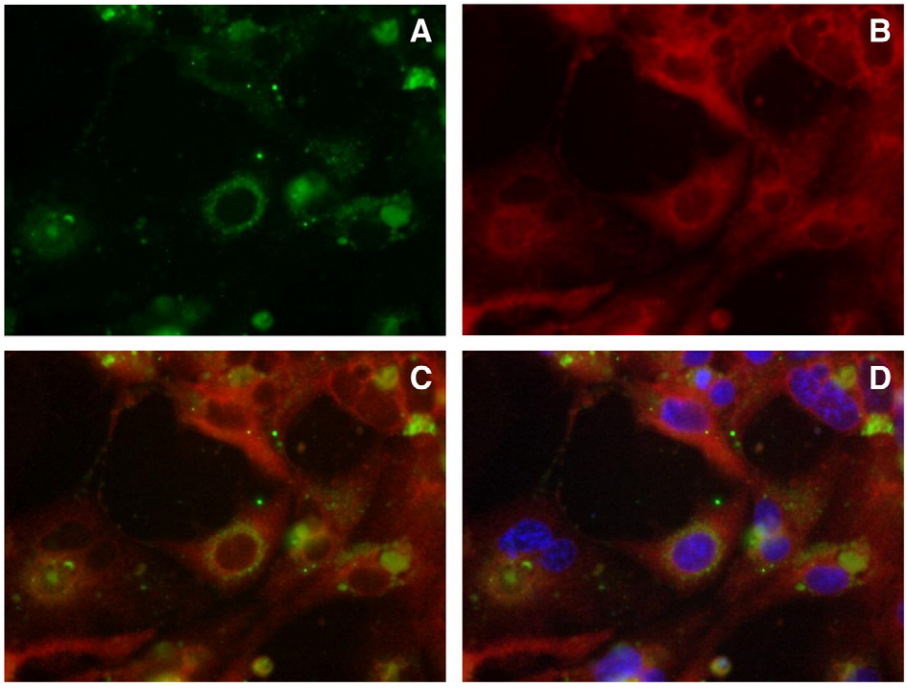

Fig. 1. Localization of exogenous α-crystallin in HLE-B3 cells. HLE-B3 cells were treated with Alexa-Fluor 488-labeled gC-αB (green) for 72 h. Cells were fixed with methanol and probed with α-crystallin antibody followed by an Alexa-Fluor-594 secondary antibody (red). Cells were mounted and stained with DAPI. Microscopy analysis of cells indicated exogenous gC-αB (A) co-localized with native α-crystallin (B) as seen when merged (C). Both protein were predominately cytoplasmic and excluded from the DAPI (blue) stained nuclei (D).

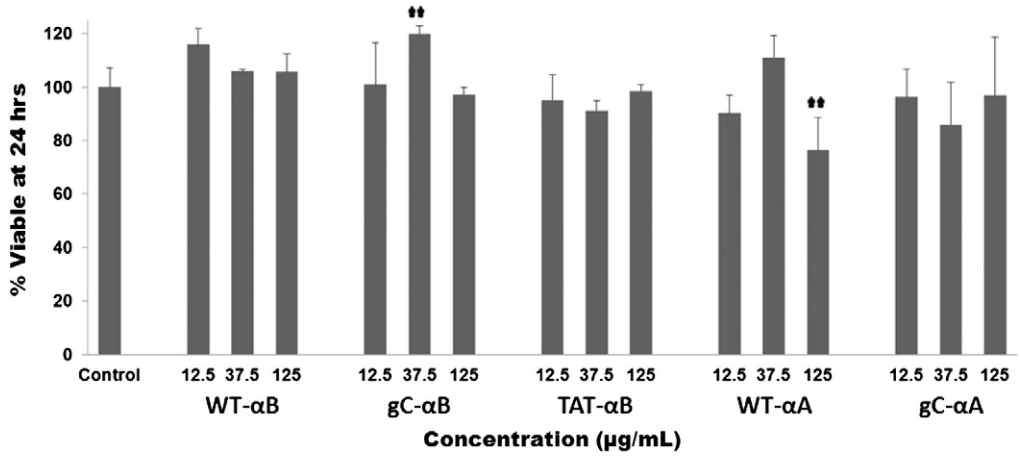

Fig. 2. Toxicity of α-crystallins on HLE-B3 cells. HLE-B3 cells were treated with increasing concentrations (12.5 μg/ml, 37.5 μg/ml, and 125 μg/ml) of WT-αB, gC-αB, TAT-αB, WT-αA, or gC-αA for 1 h. The α-crystallin proteins were removed and cells were incubated for 24 h. The viability of cells treated with α-crystallins was determined using Live/Dead Viability/Cytotoxicity Assay Kit. Untreated HLE-B3 cells fluorescence was set to 100% (control). Experiments were repeated in triplicate and reported at mean and std dev. ** = $p < 0.01$.

cell cultures exposed to intermediate concentrations of gC-αB. However, there was no dose-dependency to define a trend with either protein.

3.3. Effect of cell penetration peptide on chaperone-like activity

To determine the functional impact of modifying α-crystallins by the introduction of a cell penetration peptide (CPP), we conducted chaperone-like activity assays using two distinctly different methods to destabilize client proteins. In the first case, we examined the chaperone activity of α-crystallin proteins using heat-induced aggregation of a client protein. As in our previous studies [28], we used human aldose reductase (HAR) as a client protein to measure the ability of α-crystallin to suppress formation of light scattering aggregates. WT-αB and WT-αA almost completely prevented HAR aggregation under these conditions (Fig. 3). The gC-αB protein reduced aggregation only modestly (approximately 16%). Unexpectedly, TAT-αB and gC-αA appeared to induce additional aggregation of the HAR client protein although no apparent aggregation was observed when αA-crystallins were incubated under these conditions in the absence of client protein (not shown).

To avoid complications associated with thermal destabilization of modified α-crystallins at non-physiological temperatures, such as with the behavior of TAT-αB and gC-αA described above, we conducted additional chaperone-like activity assays at 37 °C. This was carried out by measuring the suppression of DTT-reduction induced aggregation of lysozyme as described by others [29,30]. Addition of the gC-cell penetration peptide (CPP) had virtually no effect on the chaperone-like activity of αB-crystallin. Like wild type, gC-αB can almost completely suppress lysozyme aggregation (Fig. 4). In contrast, WT-αA was somewhat less effective (prevented 61% aggregation) and gC-αA had almost no CLA activity under these conditions. Similar to our observations with the heat induced aggregation assay, there was an increase in lysozyme aggregation (114%) when chaperone-like assays were carried out using TAT-αB.

3.4. α-Crystallin-mediated protection of HLE-B3 cells against oxidative stress

We used a hydrogen peroxide oxidative stress model in HLE-B3 cells to test the ability of the various α-crystallins to protect against oxidative stress. Analysis of HLE-B3 cell viability with concentrations of H_2O_2 from 0 to 300 μM indicated that amounts of 37.5 μM or greater induced apoptosis as detected by DNA laddering and flow cytometry (data not shown). HLE-B3 cells were pretreated in media with 12.5 μg/ml recombinant α-crystallin protein for 1 h, cells were exposed to 37.5 μM H_2O_2 in media for 24 h. Using the calcein viability assay, the percent of cells protected from oxidative stress induced apoptosis by each protein was calculated (Fig. 5). All five recombinant α-crystallin proteins showed statistically significant protection of HLE-B3 cells. We observed that gC-αB was particularly effective at protecting against peroxide-induced apoptosis (cell viability was 147.0% ± 76.7%, $p < 0.001$ vs. vehicle control). However, all α-crystallins examined afforded some degree of protection against cell death, with WT-αB and its variants being somewhat more effective than αA, as WT-αA or gC-αA (Fig. 5).

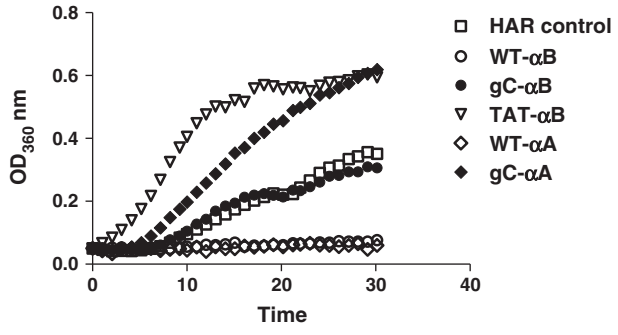

Fig. 3. Analysis of chaperone-like protection of HAR against thermal aggregation. HAR (2.5 μM) was combined with an equimolar amount of α-crystallin subunits, heated to 52 °C and monitored for protein aggregation by changes in light scattering at 360 nm. No increase in light scattering indicates protection of HAR from aggregation.

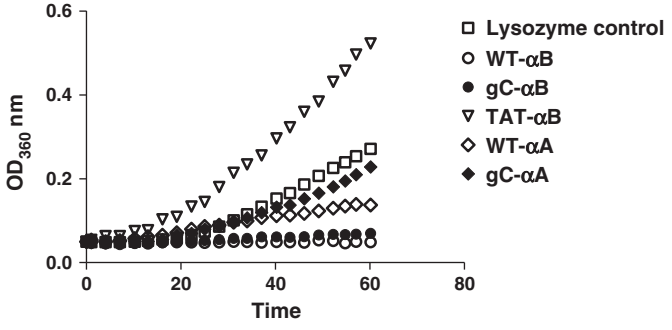

Fig. 4. Analysis of chaperone-like protection of lysozyme against DTT-induced aggregation. Lysozyme (10 μM) was combined with an equimolar amount of α-crystallin subunits and incubated for 1 h at 37 °C. Solutions were monitored for changes in light scattering by measuring absorbance at 360 nm. No increase in light scattering indicates protection of lysozyme from aggregation.

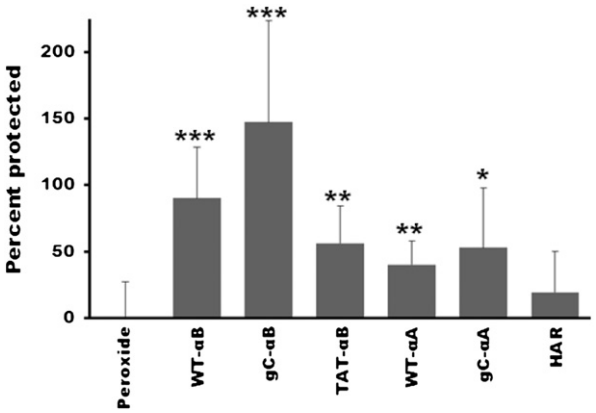

Fig. 5. Ability of recombinant α-crystallins to protect HLE-B3 cells against oxidative stress was assayed by preloading the cells using 12.5 μg/ml of α-crystallin protein (WT-αB, gC-αB, TAT-αB, WT-αA, or gC-αA) for 1 h prior to 24 h of exposure to 37.5 μM H_2O_2 followed by a calcein viability assay. Results are reported as percentage of cells protected from death as compared to the peroxide-only control. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

In all functional studies involving cell-based assays, we used recombinant HAR, purified from *E. coli* expression cultures [31], to control for possible small molecular weight contaminants derived from high density cultures of bacterial host cells. Such contaminants could possibly affect the metric being studied, especially in cell-based assays. In measurements of protection against oxidative stress, we used 170 μg HAR as a negative control for α-crystallins. Virtually no protective effect against oxidative stress was observed when HAR was substituted for α-crystallin (19.1% ± 31.0%, $p = 0.24$).

3.5. α-Crystallin-mediated protection of HLE-B3 cells against heat stress

To further test the protective effects of α-crystallin against thermal stress, we utilized a 45 °C heat-stress cell culture model of apoptosis. Cells were pre-treated with recombinant α-crystallins, and were then incubated at 45 °C for 1 h. Cell viability was measured 24 h later using the calcein assay (Fig. 6). All three αB-crystallin proteins showed a statistically significant protective effect when compared to cells receiving no protein pretreatment. The strongest protective effect occurred with WT-αB (34.0% ± 9.0%, $p < 0.001$), followed by gC-αB (32.9% ± 13.9%, $p < 0.001$) and TAT-αB (24.6% ± 14.2%, $p < 0.01$).

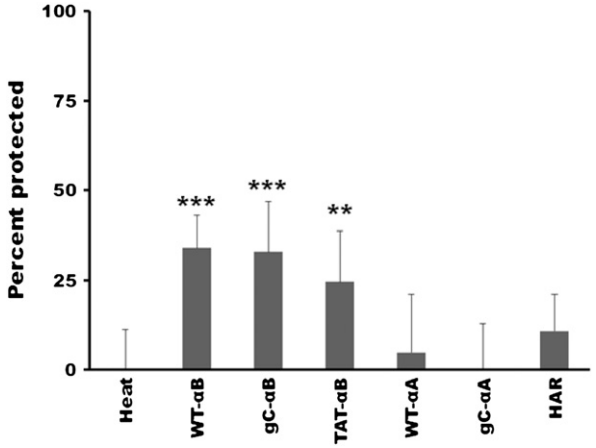

Fig. 6. α-Crystallin protection of HLE-B3 cells from heat stress. HLE-B3 cells were incubated for 1 h with 12.5 μg/ml of the indicated α-crystallins. Cells were then treated at 45 °C for 1 h. After 24 h, calcein viability assay was used to determined cell survival. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

In contrast, the αA-crystallins failed to protect HLE-B3 cells from the 45 °C heat induced apoptosis. Cells treated with both WT-αA and gC-αA did not show a statistically significant difference in cell viability (4.9% ± 16.2%, $p = 0.527$ and −2.4% ± 15.3%, $p = 0.740$, respectively). Similarly, essentially no protection was observed in cells treated with HAR (negative control) (10.9% ± 10.3%, $p = 0.084$).

3.6. Cell penetration of modified α-crystallins

α-Crystallins were labeled with an Alexa-Fluor 350 fluorescent probe to permit us to monitor cellular uptake and persistence following treatment with oxidative stress. We reasoned that this would allow us to determine if there was a correlation between α-crystallin uptake and cell viability following stress treatment. Indeed, uptake of each of the different forms of α-crystallins was easily detected by fluorescence

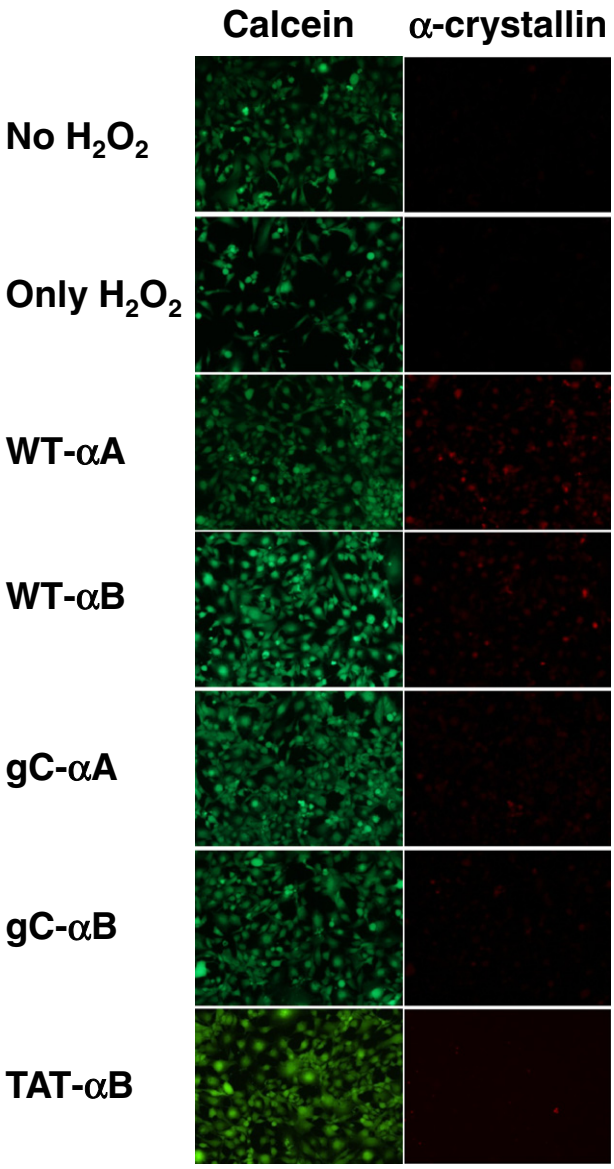

Fig. 7. Microscopy analysis of viable HLE-B3 cells treated with α-crystallin. 1 h pretreated HLE-B3 cells with Alexa-Fluor 350 labeled WT-αA, WT-αB, gC-αA, or gC-αB were followed with a chemical stress of 37.5 μM H_2O_2 exposure for 24 h or a no H_2O_2 treatment control. Viable cells were labeled and detected using calcein staining (green). Cells with exogenous α-crystallins were detected by the presence of Alexa-Fluor 350 labeled protein (red). Increased levels of α-crystallins localized with viable cells.

microscopy. We observed a correlation between cells containing Alexa-Fluor-conjugated α-crystallins and those which stained positive for calcein (green), the cell viability indicator (Fig. 7). Additionally, cells treated with α-crystallin prior to exposure to hydrogen peroxide treatment, displayed higher cell density compared to cells treated with hydrogen peroxide alone (Fig. 7). These results are consistent with the idea that the α-crystallins enter and protect cells from oxidative stress induced apoptosis.

4. Discussion

In this study, we have shown that recombinant α-crystallin can enter HLE-B3 cells and a substantial amount of it may co-localize with endogenous α-crystallins. These results provide evidence that human α-crystallins produced in a bacterial expression host and purified by conventional protein separation media can be introduced to human-derived cells without toxic effects associated with the protein or contaminating by-products. Additionally, cells treated with exogenous α-crystallin proteins had reduced levels of apoptosis when stressed with oxidative or thermal insults. This uptake of α-crystallin (Fig. 7), which occurred rapidly, correlated with protection. In the case of gC-αB, we previously found that protein uptake could be achieved within 60 min of exposure to cells [25]. Of note was the finding that even α-crystallins without cell penetration tags were able to prevent thermal and oxidative stress. Since excess α-crystallins are removed from the media prior to cells being stressed, the observation that these proteins are still protective suggests the possibility that a slower but still effective uptake mechanism is occurring. Another possibility is that α-crystallins may act through an extracellular mechanism to protect cells. This latter notion seems plausible in light of previous reports that sHSP may be associated with membranes and may be secreted from the cell [32].

We initially expected that the protective potential of α-crystallins would correlate with their in vitro chaperone-like capabilities revealed in client protein-binding assays. For example, wild type αA-crystallin, which has a strong CLA profile, was relatively ineffective in protecting against heat- and oxidative stress-induced cell death. Differences in assay conditions between in vitro and in vivo assays could explain the apparent discordancy between CLA and cellular protection. First, our chaperone-like activity assays using the heat-induced HAR aggregation assay, indicated that WT-αA and WT-αB have similarly strong chaperone activity while gC-αB and TAT-αB had minimal chaperone activity. These in vitro CLA assay results differed widely from the cell culture model results; however, the conditions for the two assays are quite different as the CLA assay was conducted at 52 °C, a temperature at which αB-crystallin has been shown to be more structurally unstable [33], while the cell culture model heat stress occurred at 45 °C. Previous studies showed that αB-crystallin undergoes heat-induced structural alterations starting at 45 °C, while αA-crystallin retains its structure to temperatures up to 55 °C [33]. It is possible that the addition of CPP tags to αB-crystallin further destabilizes the protein structure such that gC-αB and TAT-αB had substantially reduced chaperone-like activity against 52 °C heat-induced aggregation of HAR in vitro. Similarly, TAT-αB did not prevent aggregation in lysozyme DTT-induced in vitro assays, but showed a protective effect in the cell based oxidative stress model. The other α-crystallin proteins were roughly consistent between in vitro and cell based assay of oxidative stress. Furthermore, our results are consistent with previous studies in which αB-crystallin showed a stronger ability to protect against DTT-induced insulin aggregation at 35 °C than αA-crystallin. This difference disappeared at higher temperatures due to the relative instability of αB-crystallin to elevated temperatures [33].

In our chemical-induced aggregation assay, WT-αB and gC-αB exhibited the strongest chaperone-like activity. In the tissue culture model, WT-αB and gC-αB also showed the strongest ability to protect against oxidative-stress induced apoptosis. Unexpectedly, TAT-αB, which was ineffective in the lysozyme-based chaperone assays, provided significant protection to HLE-B3 cells exposed to hydrogen peroxide challenge. The reason for this divergence is unclear at this time, but may relate to translocation to subcellular compartments as reported by others [34].

All of the various forms of α-crystallin tested (WT-αB, WT-αA, gC-αB, TAT-αB, gC-αA) had protective effects on HLE-B3 cells against oxidative stress, and a subset of these (WT-αB, gC-αB, TAT-αB) also had protective effects against heat stress. αB-crystallin modified with a gC cell penetration peptide at the N-terminus (gC-αB) stood out as the most promising of the α-crystallins tested in this study. The gC-αB had strong protective effects against both heat and oxidative stress, was free of measurable toxic effects on the HLE-B3 cells, and appeared to distribute well throughout the cells as shown by immunofluorescence and Alexa-Fluor-tracking by fluorescence microscopy (Fig. 1). Others have shown that peptides derived from α-crystallin (mini-chaperone) can also prevent protein aggregation in vitro and protect cells from apoptosis [35,36].

In addition to playing a key role as a structural lens protein, it is now becoming evident that α-crystallins may contribute to protection from many diseases in the eye, including cataract, retinitis pigmentosa, and macular degeneration [35,37,38]. Protective roles for α-crystallin have been identified outside the eye as well, in conditions such as Alzheimer's disease, prion disease, autoimmune encephalomyelitis [39], and various cardiomyopathies. Prior studies to investigate the protective effects of α-crystallin by altering intracellular levels of the protein have relied on cell transfection with α-crystallin expression plasmids, as well as α-crystallin knock-out mice [40]. However, the possibility of enriching cells with recombinant α-crystallin protein supplied post-translationally received only limited study [41].

5. Conclusions

The ability to modify α-crystallins for enhanced cellular uptake lays the foundation for their use as therapeutic proteins against a wide array of diseases.

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