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Heparan sulfate mediates cell uptake of αB-crystallin fused to the glycoprotein C cell penetration peptide

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1. Introduction

αB-Crystallin, a member of the small heat shock protein (sHSP) family is highly expressed in the ocular lens [1,2]. αB-Crystallin forms high molecular weight complexes (HMWC) in the lens that undergo homo- and hetero-subunit exchange with αA-crystallin, another sHSP that has 57% sequence homology to αB-crystallin [3–5]. Early studies on αB-crystallin considered its necessary role in proper refraction; however, more recent studies have focused on its cytoprotective properties [6–9]. These studies have found αB-crystallin has chaperone-like activity (CLA) in preventing protein aggregation and anti-apoptotic activity. These properties suggest αB-crystallin could be used therapeutically to prevent intracellular protein aggregation and apoptosis.

Recent studies with αB-crystallin have been looking at its protective effects in multiple sclerosis (MS) [10,11]. These studies found levels of αB-crystallin to be highly elevated in plaques in individuals with MS. Moreover, mice with the αB-crystallin gene (*cryab*) knockout had higher levels of disease in the autoimmune encephalomyelitis model of MS [11]. Analysis of αB-crystallin has indicated that it has anti-inflammatory activity, through its CLA binding of pro-inflammatory molecules [12]. Clinical trials have indicated that systemic treatment with αB-crystallin is tolerated and shows some protective effects in reducing MS lesions activity suggesting intracellular targeting of it may provide cellular protection without genetic manipulation [13].

Cell penetration peptides (CPP) that mediate macromolecule movement across cell membranes were first reported for the HIV-1 TAT protein 25 years ago [14,15]. Further investigations narrowed the cellular uptake region to the basic amino acid region 48–60 [16]. A number of other studies have identified other CPPs that mediate protein uptake into cells [17]. These CPPs are reported to bind glycosaminoglycans (GAG) of target cells allowing for macromolecule uptake. Additionally, arginine residues within these CPPs have been shown to interact with membrane phospholipids and mediate direct translocation of macromolecules [18]. The ability of CPPs to mediate protein uptake into cells allows for the possibility to deliver αB-crystallin into cells.

Previously we reported the fusion of CPPs to αB-crystallin in which these fusion proteins consisted of the TAT peptide or the gC peptide, derived from the herpes simplex virus type I (HSV-1) glycoprotein C (gC) [19]. These bacterial expressed CPP-tagged recombinant proteins had enhanced cellular uptake compared to wildtype αB-crystallin (WT-αB). However, only the gC fused αB-crystallin (gC-αB) displayed chaperone activity. In addition, gC-αB has been shown to protect cells from thermal and chemically induced apoptosis [20]. Analysis of protein uptake in organ culture indicated that mixtures of gC-αB with αA-crystallin gave enhanced uptake of the latter into the ocular lens [21]. However, the route of protein uptake in cells and organ culture was not determined.

In this study, we analyzed the ability of gC-αB to be taken up by cells deficient in glycosaminoglycans (GAGs) and whether removal of heparan sulfate (HS) affects protein uptake. Additionally, we constructed four gC-αB mutants that have mutations of one to four amino acids of the gC peptide. These mutants were also analyzed for protein uptake in GAG deficient cells. Analysis of gC-αB uptake by multiple cell types indicated a binding of HS, which when removed by genetic or enzymatic modification results in decreased gC-αB uptake. Moreover, a cysteine to alanine substitution indicated no loss in gC-αB uptake or anti-apoptotic activity. However, unlike unmodified gC, it did not form disulfide bridges. This finding could conceivably make it a better CPP as it would not cross link fusion proteins potentially diminishing functional activity.

2. Methods

2.1. Construction of recombinant human αB-crystallin containing CPP

DNA primer oligomers for each of the four mutant gC CPPs were designed with *Nco*I sequences and obtained from IDT (Coralville, IA). PCR was performed using Table 1 primers with our previously described reverse primer and gC-αB expression construct [19]. PCR reactions were for 30 cycles of 95 °C, 50 °C, and 72 °C for 1 min at each step. Resulting DNA fragments were isolated on a 1% agarose Tris-acetate EDTA (TAE) gel. DNA fragments of the expected size were isolated from the gel and purified using the quick spin kit (Qiagen, Germantown, MD) into 30 μL of 50 mM Tris-HCl pH 8.5. Isolated DNA

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Table 1
Oligonucleotides sequences used for PCR and cloning.

Name	Sequence (5′ to 3′)[a]
αB Reverse	AACAAGCTTTCATTTCTTGGGGGCTGC
Mut A For	TTTCCATGGGATCACGAGTGCAAATCCGATGTCGATTTCGAAATACCTCACGAGACATCGCC
Mut B For	TTTCCATGGGATCAAAAGTGCAAATCAAATGTAAGTTTAAAAATTCAACCCGAGAC
Mut C For	TTTCCATGGGATCACGAGTGCAAATCCGAGCTCGATTTCGAAATTCAACCCGA
Mut D For	TTTCCATGGGATCACGAGCGCAAGCCCGATGTCGATTTCGAGCTTCAACCCGAGACATC

[a] Restriction endonuclease recognition sites are underlined.

fragments along with pET-23d vector DNA were digested with *Nco*I and *Hin*dIII (New England Biolabs, Ipswich, MA). Restriction fragments were resolved by 1% agarose TAE gel electrophoresis and DNA bands excised. Purified PCR products along with pET-23d vector DNA were ligated together using quick ligase kit (NEB) and transformed into *E.coli* strain TOP 10 (Thermo Fisher, Waltham, MA). Bacterial colonies were selected and inserts confirmed by DNA sequencing.

2.2. Production of recombinant α-crystallin proteins

For all expression constructs, plasmids were transformed into *E.coli* strain BL 21(DE3) cells (Thermo Fisher). Starter cultures of 25 mL were grown overnight. Expression cultures were performed in 2 × 400 mL of M9CA and 100 μg/mL ampicillin and inoculated with 8 mL of starter culture, as described previously [19–22]. Cultures were grown for 4 h at 37 °C to an OD_{600} = ~0.7. Bacterial cultures were induced with a final concentration of 1 mM IPTG and protein expression was allowed to proceed overnight at 37 °C. *E. coli* were harvested by centrifugation at 10,000 x g for 15 min. The bacterial pellet was suspended in 50 mL of Phosphate buffered saline (PBS) with 10 mM $MgCl_2$, 1 mM MnCl and DNase I (Sigma-Aldrich, St Louis, MO) and lysed by three passages in a French press (Thermo Fisher) at 1500 psi. Cell lysates were centrifuged at 15,000 × *g* for 30 min at 4°C. Soluble protein fractions were loaded onto Histrap FF column 4 × 1 mL (GE healthcare, Waukesha, WI) charged with cobalt (gC-αB) or nickel (mutants A-D). Following a column wash with 20 mL of PBS, proteins were eluted with 150 mM Imidazole in PBS. Peak fractions positive for α-crystallin proteins were further purified by gel-filtration using an Enrich SEC 70 10 × 300 column (Bio-Rad) and eluted with PBS. Fractions enriched for α-crystallin were analyzed by 4–20% 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.

2.3. Reducing and non-reducing SDS-PAGE

Purified α-crystallin proteins were mixed with 2× SDS sample loading buffer and 1% 2-mecaptoethanol. Samples were heated at 95°C for 5 min, loaded onto 4–20% mini protean TGX stain free gels (Bio-Rad) and separated by SDS-PAGE at 200 V for 30 min. Gels were imaged using ChemiDoc XRS+ system (Bio-Rad). For non-reducing conditions, 1% 2-mercaptoethanol was omitted from samples.

2.4. Protein conjugation to Alexa-Fluor-488

Proteins were conjugated with Alexa-Fluor 488 fluorophores according to the manufacturer's protocol (Thermo Fisher). Concisely, αB-crystallin preparations in PBS were mixed with 100 mM sodium bicarbonate Alexa-Fluor 488 dye added and incubated in the dark for 1 h at 25 °C·Labeled α-crystallins were dialyzed against 1 L of PBS overnight to remove excess label. The concentrations of α-crystallin proteins were determined and stored at 4 °C until used.

2.5. Microscopy analysis of protein uptake

Wildtype Chinese Hamster Ovary (CHO) cells were a gift from Dr. Gregory P. Owens (UC-Denver) and GAG deficient CHO cell lines (CRL-2242 and CRL-2244) were purchased from ATCC (Manassas, VA) and confirmed by STR analysis (CU Denver sequencing core). Cells were plated on 18 mm glass coverslips (Matsunami, Bellingham, WA) in 12-well tissue culture plates at a density of 1×10^5 cells. Cells were allowed to adhere in standard media (Ham's Modified F-12,1× Penicillin/Streptomycin, 10% Fetal Bovine Serum (FBS)) overnight. Standard media was removed and replaced with 400 μL serum free media (Thermo Fisher) along with either 20 μM Alexa-Fluor 488 conjugated protein or PBS for 1 h at 37 °C. For the last 30 min of this procedure, 1 μg/mL Hoechst 33342 (Sigma-Aldrich) was added to label nuclei. Coverslips were washed three times in PBS and mounted on frosted slides in Vectashield Hardset Antifade Mounting medium (Vector Labs, Burlingame, CA). Images were captured on a laser scanning confocal microscope (C2+; Nikon Instruments, Inc., Melville, NY) to acquire 1024 × 1024 pixel photographs one channel at a time with either a 20× air or 40× oil objective. Ten fields of each cell type at equivalent cell densities were collected. Images were split in NIH Image J to show the green channel only before converting to binary mode. Pixels ≥5 were counted by the software for each image. Each counted pixel was measured for intensity by NIS-Elements AR 4.20 software (Nikon).

2.6. Flow cytometry analysis of protein uptake

For flow cytometry analysis, experiments were performed with a fetal human lens epithelial cell line (FHL-124 cells) to better mimic human lens cells and not genetically modified hamster cells. FHL-124 cells were plated at a density of 5×10^5 cells per well in a 6-well plate and grown in DMEM + Penicillin/Streptomycin +10% FBS and allowed to attach overnight. Alexa-Fluor 488 labeled αB-crystallin (5 μM or 50 μM) or PBS control was incubated for 1 h at 37 °C in 400 μL serum free media (Thermo Fisher). Cells were then washed twice with serum free media and incubated for an additional 24 h. For heparinase treatment experiments, 2 U/mL of heparinase III (Sigma) was added 2 h prior to αB-crystallin incubation. For flow cytometry analysis cells were detached with a 5 min treatment with 0.25% trypsin, washed in PBS and suspended in 10 mM Hepes (pH 7.4), 140 mM NaCl, and 2.5 mM $CaCl_2$ solution with or without 1 μg/mL Hoechst 33342. At least 15,000 singlet events per treatment were counted by gating on on cells using forward and side scatter, then Hoescht 33,342 positivity on a Beckman Coulter Gallios (Brea, CA) flow cytometric analyzer in the UC Cancer Center Core. Data from triplicate experiments are shown.

2.7. Determination of high molecular weight complexes

Purified α-crystallin proteins were loaded onto an Enrich SEC 650 10 × 300 column using an NGC Quest Chromatography system (Bio-Rad, Hurcules, CA). Proteins were eluted with PBS into 1 mL fractions. The elution profiles of α-crystallins were monitored by absorbance (280 nm) and plotted against size standards including thyroglobulin,