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α B-Crystallin/sHSP Protects Cytochrome c and Mitochondrial Function Against Oxidative Stress in Lens and Retinal Cells

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Abstract

Background— α B-crystallin/sHSP protects cells against oxidative stress damage. Here, we mechanistically examined its ability to preserve mitochondrial function in lens and retinal cells and protect cytochrome c under oxidative stress conditions.

Methods— α B-crystallin/sHSP was localized in human lens (HLE-B3) and retinal (ARPE-19) cells. α B-crystallin/sHSP was stably over-expressed and its ability to preserve mitochondrial membrane potential under oxidative stress conditions was monitored. Interactions between α B-crystallin/sHSP and cytochrome c were examined by fluorescent resonance energy transfer (FRET) and by co-immune precipitation. The ability of α B-crystallin/sHSP to protect cytochrome c against methionine-80 oxidation was monitored.

Results— α B-crystallin/sHSP is present in the mitochondria of lens and retinal cells and is translocated to the mitochondria under oxidative conditions. α B-crystallin/sHSP specifically interacts with cytochrome c *in vitro* and *in vivo* and its overexpression preserves mitochondrial membrane potential under oxidative stress conditions. α B-crystallin/sHSP directly protects cytochrome c against oxidation.

Conclusions—These data demonstrate that α B-crystallin/sHSP maintains lens and retinal cells under oxidative stress conditions at least in part by preserving mitochondrial function and by protecting cytochrome c against oxidation. Since oxidative stress and loss of mitochondrial function is associated with eye lens cataract and age-related macular degeneration, loss of these α B-crystallin/sHSP functions likely plays a key role in the development of these diseases.

General Significance— α B-crystallin/sHSP is expressed throughout the body and its ability to maintain mitochondrial function is likely important for the prevention of multiple degenerative diseases.

Keywords

Cytochrome c; Oxidation; α B-crystallin; sHSP; Mitochondria

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1. Introduction

α B-crystallin/sHSP is a member of the small heat-shock (sHSP) family of proteins [1] that are characterized by having molecular chaperone-like activity [2, 3]. sHSPs are among the most evolutionarily conserved of all proteins and form large multi-subunit complexes that are capable of sequestering and detoxifying denatured and aggregated proteins [4, 5, 6].

In addition to α B-crystallin/sHSP, the sHSP superfamily also includes HSP27 [7] and α A-crystallin/sHSP [8] among others. α B-crystallin/sHSP and HSP27 are expressed in most tissues while α A-crystallin/sHSP is mainly expressed in the eye lens where it is found as a complex with α B-crystallin/sHSP to form total α -crystallin/sHSP which consists of one α B-crystallin/sHSP subunit to three α A-crystallin/sHSP subunits. α -crystallin/sHSP is an important structural protein in the eye lens that comprises up to 40% of the total protein of the lens [9].

In addition to the lens, α B-crystallin/sHSP is found at high levels in the heart where it constitutes as much as 5% of total heart protein [10]. It is also present at high levels in the brain [11], muscle [12, 13], and many parts of the eye including the retinal photoreceptors [14], the retinal pigmented epithelium (RPE) [15], Bruch's membrane [16], and the trabecular meshwork [17]. α B-crystallin/sHSP has been localized to multiple sub-cellular structures and components in multiple cell types including the cytosol [18], nucleus [19], Golgi apparatus [20], cytoskeleton [21, 22] and, the mitochondria [18]. Since the majority of these cells are long-lived and exhibit low protein turn-over, the chaperone-like activity of α B-crystallin/sHSP has been proposed to protect cells against insult and be important for cellular longevity [2]. However, the many proteins protected by α B-crystallin/sHSP and the cellular functions it preserves have yet to be fully elucidated.

α B-crystallin/sHSP can protect proteins against thermal [2, 23] and chemical [2, 3] denaturation *in vitro*, and it can preserve cellular viability under thermal [24], osmotic [25] and, oxidative stress conditions [26]. Indeed, a specific mutation in α B-crystallin/sHSP that diminishes its chaperone-like activity [27] is associated with desmin-related myopathy, an autosomal dominant disorder characterized by desmin deposition in muscle fibers [28, 29]. α B-crystallin/sHSP chaperone function loss is also associated with eye lens cataract formation [30].

α B-crystallin/sHSP has been implicated as a key player in defense of cells against oxidative stress damage. It has been shown to defend cells against oxidative stress-induced loss of protein function [31, 32], disruption of cytoskeletal assembly [33, 34] and apoptotic induction [28, 35-43]. It has been shown to preserve cellular viability upon oxidative stress exposure in multiple cells and tissues including glial cells [44], heart [45], retina [18] and kidney [46]. It has also been shown to protect tissues against peroxide-induced damage [18], UV-light damage [38] and damage due to ischemic-reperfusion damage [47]. The importance of α B-crystallin/sHSP for oxidative stress defense is underscored by its highly altered expression patterns in a multitude of oxidative stress-associated diseases including Parkinson's disease [48], Alexander's Brain Disease [49], Lewy Body Disease [50, 51], Alzheimer's Disease [52, 50], Huntington's Disease [53], Creutzfeldt-Jakob disease [54], Desmin-Related Myopathy [29], age-related macular degeneration [16, 55, 56], and age-related cataract [57, 58]. Despite its ability to confer oxidative stress protection to cells the cellular targets and functions protected by α B-crystallin/sHSP under oxidative stress conditions have not been entirely established.

One key cellular function of α B-crystallin/sHSP associated with oxidative stress protection is its ability to prevent apoptosis through modulation of apoptotic control proteins [35-39, 59]. A major regulator of apoptosis is the mitochondrial electron transport protein

cytochrome c (cyt c) that initiates apoptosis upon its oxidation at methionine-80 (met-80) and its subsequent release from the mitochondria into the cytosol [75]. Cyt c is a 12 kDa oxido-reductase protein that functions to transfer electrons from complex III to complex IV of the electron transport chain [61]. Multiple studies have demonstrated that oxidation of cyt c at met-80 results in a loss of cyt c electron transport function [62, 63]. Oxidation of cyt c at met-80 induces a conformational change that initiates mitochondrial release and induction of apoptosis [61, 63-65, 75].

It has been recently shown that mitochondrial function is essential for lens [66, 67] and RPE cell viability [68] since deletion of the mitochondrial antioxidant enzyme MsrA results in loss of lens and RPE viability and mitochondrial function. MsrA has been demonstrated to repair oxidized met-80 in cyt c restoring its electron transport function. These data provide evidence that maintenance of lens and RPE cell cyt c oxidation state is critical for the mitochondrial function and viability of lens and retinal cells.

It has recently been demonstrated that α B-crystallin/sHSP is translocated from the cytosol to the mitochondria upon chemical and oxidative stress of RPE and heart cells [69, 70] suggesting that α B-crystallin/sHSP could be important for one or more mitochondrial functions in lens and RPE cells including protection of cyt c against met-80 oxidation and thus maintenance of mitochondrial function. To test this hypothesis, we localized α B-crystallin/sHSP to the mitochondria of human lens epithelial and RPE cells, we measured the translocation of α B-crystallin/sHSP to the mitochondria of these cells under oxidative conditions, we tested for interactions between cyt c and α B-crystallin/sHSP and we correlated these data with the ability of α B-crystallin/sHSP to protect cyt c against met-80 oxidation *in vitro* and preserve mitochondrial membrane potential *in vivo*.

Our data confirm that α B-crystallin/sHSP is indeed localized to the mitochondria of lens and RPE cells and translocates to the mitochondria in these cells under oxidative stress conditions. We also demonstrate that α B-crystallin/sHSP protects the mitochondrial function of lens and retinal cells exposed to oxidative stress and show that α B-crystallin/sHSP interacts with and protects cyt c against met-80 oxidation.

On the basis of this data, we propose that α B-crystallin/sHSP functions, at least in part, to protect lens and RPE cells against oxidative stress damage by protecting cyt c against oxidation and thereby preserving mitochondrial function. Since oxidative stress and loss of lens and RPE mitochondrial function are associated with the development of eye lens cataract [71] and age-related macular degeneration [71], we propose that the ability of α B-crystallin/sHSP to preserve mitochondrial function in the lens and the RPE may be important for the prevention of these and potentially other age-related degenerative diseases.

2. Materials and Methods

2.1 Cell culture

Human lens epithelial cells (HLE-B3) immortalized by infection with adenovirus 12-SV40 [72] and human retinal pigmented epithelial cells (ARPE-19) were grown and cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 15% FBS (Invitrogen), gentamicin (50 units/ml; Invitrogen), penicillin-streptomycin antibiotic mix (50 units/ml; Invitrogen) and fungizone (5 μ l/ml; Invitrogen) at 37 °C in the presence of 5% CO₂.

2.2 Localization of α B-crystallin/sHSP to the mitochondria in human lens and retinal cells

Lens and RPE cells were plated onto coverslips in 12 well plates at a density of 200,000 cells per well and incubated overnight in complete media. Standard procedures were used for double immunofluorescence staining. Briefly, cells were fixed with 3.7%

paraformaldehyde in PBS, permeabilized with 0.25% TritonX-100 in PBS, and blocked with 1% BSA. To investigate whether α B-crystallin/sHSP localizes to the mitochondria, lens and RPE cells were stained with Mitotracker Red CMXRos (Molecular Probes, Invitrogen) for 45 min at 250 nM as indicated by the manufacturer's protocol. Following Mitotracker red staining, rabbit polyclonal anti- α B-crystallin antibody (Stressgen), was incubated overnight in 4 °C at a 1:100 dilution. Cells were washed three times with PBS, and then incubated with Alexa Fluor 488 goat anti-rabbit secondary antibody (1:2,000) for one hour at room temperature. Cells were washed three times with PBS, and mounted onto slides using ProLong Gold Antifade Reagent (Invitrogen). Immunofluorescence staining was visualized using a Zeiss LSM700 confocal microscope.

To analyze the mitochondrial localization under oxidative stress conditions, lens and RPE cells were incubated for 2 h in serum free DMEM followed by treatment with either 0 μ M, 100 μ M or 200 μ M H₂O₂ for 1 h. After treatment, cells were immediately harvested and mitochondrial and cytosolic fractions were isolated and analyzed by analyzed by SDS-PAGE and western blotting.

2.3 Isolation of cytosolic and mitochondrial proteins

Mitochondria were isolated from lens and RPE cells using a mitochondrial isolation kit for cultured cells (Mitoscience) according to the manufacturer's protocol. Briefly, lens and RPE cells were detached by trypsinization and centrifuged at 1,000 g for 3 min. The pellet was frozen at -80 °C to weaken the cell membrane. Cells were re-suspended in Reagent A and homogenized for 30 strokes in a pre-chilled dounce homogenizer. The homogenate was centrifuged at 1,000 g for 10 min. The supernatant was removed and the pellet was resuspended in Reagent B, homogenized, and centrifuged at 1,000 g for 10 min. The combined supernatants were further centrifuged at 12,000 g for 15 min. The resulting supernatant was collected as the cytosolic fraction and the mitochondrial pellet resuspended in Reagent C. Protein concentrations were determined by Bradford protein assay with BSA as a standard as previously described [66].

2.4 SDS-PAGE and western blotting

Unless specified, all electrophoresis reagents and apparatus were purchased from Bio-Rad (Richmond, CA). Protein samples were mixed with 2X laemmli sample buffer at a 1:1 ratio and heated to 100 °C for 5 min. The samples were separated by electrophoresis on a 15% sodium dodecyl sulfate (SDS) gel. Proteins were transferred onto nitrocellulose membranes (Amersham-Pharmacia, Piscataway, NJ) using a Mini Trans-Blot electrophoresis transfer cell apparatus (Bio-Rad). The membrane was equilibrated in Tris-buffered saline (TBS), pH 7.4, 0.05% Tween-20 for 15 min then blocked in TBS (pH 7.4), 5% Carnation nonfat milk, and 0.05% Tween-20, for 1 h. The membrane was incubated with the appropriate antibody overnight, followed by incubation for 1 h with the appropriate secondary antibody (GE Healthcare). Immunoblots were visualized using ECL western blotting reagents (Amersham-Pharmacia) as specified by the manufacturer. The following antibody concentrations were used where appropriate: primary anti-cyt c (R & D) 1:5,000, primary anti- α B-crystallin (Stressgen) 1:20,000, secondary anti-mouse (cyt c) 1:20,000 or anti-rabbit (α B-crystallin) 1:5,000.

2.5 Production of fluorescently tagged protein vectors and fluorescence resonance energy transfer (FRET)

pAcGFP-C1 and pDsRED Monomer-C1 vectors were purchased from Clontech (Palo Alto, CA). pAcGFP1-C1 vector is encoded with green fluorescent protein (GFP- $\lambda_{ex}/\lambda_{em}$ =475/505 nm). The pDsRED-Monomer-C1 is encoded with a red fluorescent protein DsRED ($\lambda_{ex}/\lambda_{em}$ =557/585 nm). PCR reactions were performed to insert restriction sites onto the 5' and

3' ends of the insert cDNAs using primers listed in Table 1, the cyt c gene was subcloned into pAcGFP-C1, and the α B-crystallin/sHSP gene subcloned to pDsRED monomer-C1 terminals using restriction digest and ligation reactions. A GFP-dsRED fusion vector was also prepared for use as a positive control by sub-cloning dsRED cDNA from pDsRED Monomer-C1 into pAcGFP-C1. Successful cloning was confirmed by automated sequencing. Plasmids were transfected into lens and RPE cells using Lipofectamine 2000 transfection reagent (Invitrogen) according to manufacturer's instructions. Positive FRET emissions were detected by exciting transfected cells at the optimal excitation wavelength of 488 nm for the donor GFP and FRET emissions were observed by way of emission from the acceptor, dsRED, at 585nm.

2.6 Transfection of cytochrome c-GFP and α B-crystallin-dsRED in lens and RPE cells and localization to the mitochondria

Lens and RPE cells were plated onto coverslips in 12 well plates at a density of 200,000 cells per well and incubated overnight in complete media. Cells were transfected with either the cytochrome c-GFP (cyt c-GFP) vector or the α B-crystallin-dsRED (α B-dsRED) vector using Lipofectamine 2000 (Invitrogen) following manufacturer's instructions. To localize cyt c-GFP to the mitochondria, lens and RPE cells were stained with 250 nM Mitotracker Red CMXRos (Molecular Probes, Invitrogen) for 30 min as indicated by the manufacturer's protocol. Cells were then fixed with 3.7% paraformaldehyde in PBS and visualized using the Zeiss LSM700 confocal microscope. For localization of α B-dsRED to the mitochondria the cells were first fixed with 3.7% formaldehyde in PBS then stained with 200 nM MitoView Green (Biotium, Hayward, CA) for 30 min according to manufacturer's instructions. Cells were visualized using the Zeiss LSM700 confocal microscope.

2.7 Production of α B-crystallin-overexpressing lens and RPE cell lines

Stable α B-crystallin/sHSP overexpressing lens and RPE cell lines were developed using the ViraPower Lentiviral Expression System (Invitrogen), utilizing the pLenti6/V5-D-Topo plasmid according to the manufacturer's instructions. Primers were designed to amplify full length α B-crystallin transcripts. The resulting inserts were cloned into the expression vector, sequenced to ensure authenticity and correct orientation, and subsequently used to transfect HEK293-FT kidney cells for production of the viral construct. Virus particles were harvested from the HEK293-FT cells and used to infect lens and RPE cells. α B-crystallin/sHSP overexpressing cells were selected by blasticidin treatment (Invitrogen). The overexpression of α B-crystallin/sHSP in lens and RPE cells was confirmed by RT-PCR analysis.

2.8 Analysis of mitochondrial membrane potential (MMP)

Control and α B-crystallin/sHSP overexpressing lens and RPE cells were seeded at a density of 200,000 cells/dish on 35 mm glass bottomed dishes. The cells were incubated in serum free media for 2 h before treatment with H₂O₂ at either 0 μ M or 50 μ M for 24 hrs. For mitochondrial membrane potential analysis, the cells were stained with 5 μ M JC-1 (Invitrogen) for 20 min. MMP changes were examined using the Zeiss LSM700 confocal microscope by observing a red to green color shift which resulted in an increase in yellow emission in the merged images, indicating decreased MMP.

2.9 Analysis of α B-crystallin/sHSP transcripts in α B-crystallin/sHSP overexpressing cells

RNA was isolated from both α B-crystallin/sHSP overexpressing as well as control lens and RPE cell lines using the TRIzol® method (Invitrogen). Cells were harvested in 1 mL TRIzol® reagent per 10 cm² culture dish. Samples were incubated for 5 min at room temperature, 0.2 mL of chloroform was added, and sample were shaken vigorously for 15 s

followed by incubation at room temperature for 2–3 min. Samples were centrifuged at 12,000 g for 15 min at 4 °C to separate the mixture into a lower red phenolchloroform phase, an interphase, and a colorless upper aqueous phase. RNA remained exclusively in the upper aqueous phase. The aqueous phase was carefully removed and RNA precipitated by adding 0.5 mL of 100% isopropanol, incubated at room temperature for 10 min and centrifugation at 12,000 g for 10 min at 4 °C. The RNA pellet was then washed twice with 1 mL of 75% ethanol and centrifuged at 7,500 g for 5 min at 4 °C before air-drying for 5–10 min. The pellet was resuspended in RNase free water. α B-crystallin/sHSP transcripts were evaluated by semi-quantitative RT-PCR using the SuperScript® III one-step RT-PCR system with PlatinumTaq polymerase (Invitrogen) according to the manufacturer's instructions. GAPDH was amplified as the internal control transcript for 30 PCR cycles with a 60 °C annealing temperature and the primer sequences: Forward 5' - CCACCCATGGCAAATTCCATGGCA-3' and reverse primer 5' - TCTAGACGGCAGGTCAGGTCCACC-3'. α B-crystallin/sHSP transcripts were amplified for 25 cycles with a 57 °C annealing temperature and the primer sequences: Forward primer 5' - AGCCGCCTCTTTGACCAGTTCTTC-3' and reverse primer 5' - GCGGTGACAGCAGGCTTCTCTTC-3'.

2.10 Immunoprecipitations

1 mg of lens and RPE whole cell lysate was incubated with 2 μ g anti-cytochrome c antibody (Abcam) or 4 μ g anti- α B-crystallin antibody (Stressgen) for 2 h at 4 °C while rotating. 25 μ l of packed Protein A-agarose beads (100 μ l bead matrix; Sigma, St, Louis, MO) was added and samples mixed by rotation at 4 °C overnight. The beads were then collected by centrifugation at 12,000 rpm for 30 s in a microcentrifuge. The supernatant was saved for further analysis. Beads were washed 3 times in 500 μ l RIPA buffer. Protein was eluted from the beads by boiling for 5 min in 40 μ l 2X SDS loading buffer. Eluted protein was analyzed by SDS-PAGE and western blotting using 4–20% Mini-PROTEAN® TGX™ Gels (Bio-Rad).

2.11 Cloning, expression, and purification of WT α B-crystallin/sHSP

Human α B-crystallin cDNA in a pET-20b(+) expression vector (a gift from Mason Posner) was used to produce recombinant α B-crystallin/sHSP protein. Sequences were confirmed by automated sequencing and the vectors transformed into BL21 (DE3) competent E. coli (Invitrogen). Protein expression was induced using Isopropyl- β -D-thio-galactoside (IPTG). α B-crystallin/sHSP protein was purified by gel filtration chromatography using a Sephacryl G300 packed column, fractions were collected and concentrated using an Amicon® Ultra-15 centrifugal filter device with a nominal molecular weight cutoff of 10 kDa. Protein purity was confirmed by SDS-PAGE and concentrations measured using a Bradford protein assay with BSA as a standard as previously described [66].

2.12 Peroxidase assay

Oxidized cyt c was prepared by treating purified horse heart cyt c protein (Sigma-Aldrich) with HOCl at a 12:1 molar ratio (HOCl:cyt c) for 15 min and the enzymatic peroxidase activity was measured by ABTS oxidation [73]. The effect of oxidation on the peroxidatic activity of cyt c was assayed by adding 0.6 μ M cyt c to 1 ml of the mixture containing 1.3 mM 2,2-azino-bis(3-ethylbenzthiozoline-6-sulfonic acid) diammonium salt (ABTS), 12 mM H₂O₂, and 100 μ M diethylenetriaminepentaacetic acid (DEPTA) in 100 mM phosphate buffer (pH 7.4) at 23 °C, followed by measuring the increase in absorbance at 420 nm over 60 s [63]. To measure the protection of cyt c by α B-crystallin/sHSP the reaction was repeated in the presence of α B-crystallin/sHSP at a 1:1 w/w ratio to cyt c.

2.13 Confirmation of methionine-80 oxidation in cytochrome c

Met-80 oxidation of cyt c was confirmed by measuring the absorbance of cyt c at 695 nm [63]. Reduced met-80 in cyt c resulted in an absorbance spectra of cyt c with a peak at 695 nm, however oxidized cyt c containing oxidized met-80 shows a decrease in the 695 nm peak. This effect has been attributed to the oxidation of the met-80 ligand [76]. The absorbance of 0.17 mM purified horse heart cyt c protein in 50 mM sodium phosphate pH 7.4, was measured at 695 nm. Cyt c oxidation with HOCl was performed as described above (12:1 HOCl:protein molar ratio) and the absorbance at 695 nm measured after 15 min. To measure the protection of the met-80 α B-crystallin/sHSP was added to cyt c at 1:1 w/w ratio prior to HOCl treatment.

3. Results

3.1 α B-crystallin/sHSP is present in the mitochondria of human lens and RPE cells

Translocation of α B-crystallin/sHSP to the mitochondria has previously been shown in mouse heart and RPE cells exposed to oxidative stress [69, 70] suggesting an important function for α B-crystallin/sHSP in mitochondrial protection against oxidative stress. To establish a potential role for α B-crystallin/sHSP in mitochondrial protection of lens and retinal cells exposed to oxidative stress, we first established the subcellular localization pattern of α B-crystallin/sHSP in lens and RPE cells by immunofluorescent staining with an α B-crystallin-specific antibody and by western blotting of cytosolic and mitochondrial fractions.

Immunofluorescent staining revealed (Fig. 1) that α B-crystallin/sHSP is localized to the mitochondria and the cytosol of both lens and RPE cells. As shown in Fig. 1A, α B-crystallin/sHSP (green) co-localizes with the mitochondrial-specific marker Mitotracker Red (red) in both lens and RPE cells as indicated by the merged images (orange). Western blot analysis of cytosolic, mitochondrial and total cell protein extracts from the lens and RPE cells confirmed mitochondrial and cytosolic localization of α B-crystallin/sHSP (Fig. 1B).

3.2 dsRED-tagged α B-crystallin/sHSP localizes to the mitochondria of human lens and RPE cells

To further establish the localization of α B-crystallin/sHSP in lens and RPE cells, cells were transiently transfected with a fluorescently tagged α B-crystallin fusion protein (α B-dsRED) and the localization of the tagged protein was examined by confocal microscopy. As shown in Fig. 2A, α B-dsRED (red) co-localizes in lens and RPE cells with the mitochondrial specific marker Mitoview Green (green) as indicated by the merged image (yellow/orange). As a control, lens and retinal cells were also transiently transfected with fluorescently tagged cyt c fusion protein (cyt c-GFP). As expected, cyt c-GFP (green) co-localizes with the mitochondrial specific marker Mitotracker Red (red) as indicated by the merged image (yellow/orange) (Fig. 2B). Not all the cells were transfected as evidenced by the presence of non-fluorescent (lacking red or green) cells.

3.3 α B-crystallin/sHSP translocates to the mitochondria of human lens and RPE cells upon oxidative stress treatment

It has previously been demonstrated that α B-crystallin/sHSP translocates to the mitochondria of heart cells during *ex vivo* ischemia and in CoCl₂-treated retinal cells [69]. To determine if mitochondrial translocation also occurred upon H₂O₂-treatment of lens and RPE cells the cells were treated for 1 hour with H₂O₂ and mitochondrial and cytosolic cellular fractions were subsequently isolated. SDS-PAGE and western blot analysis (Fig. 3) revealed that α B-crystallin/sHSP levels increased in the mitochondrial fractions of both lens (Fig. 3A) and RPE (Fig. 3B) cells upon H₂O₂ oxidative stress treatment. Cytosolic levels of

α B-crystallin/sHSP appeared to remain constant in the lens cells (Fig. 3A), however there was a decrease in the cytosolic levels of α B-crystallin/sHSP in the RPE cells (Fig. 3B). Colloidal blue staining demonstrates equal protein loading. The reason for the steady levels of cytosolic α B-crystallin/sHSP in the lens cells is not known.

3.4 Overexpression of α B-crystallin/sHSP preserves mitochondrial membrane potential in lens and retinal cells exposed to oxidative stress

Based on the mitochondrial localization (Fig. 1 and 2) and oxidative stress-induced translocation of α B-crystallin/sHSP in lens and retinal cells (Fig. 3), we sought to determine if over-expression of α B-crystallin could provide protection of lens and retinal cell mitochondrial function upon oxidative stress treatment. We examined mitochondrial membrane potential (MMP) by JC-1 staining in cells exposed to H₂O₂-stress [74]. JC-1 exists in the mitochondria as an aggregate emitting red fluorescence and in the cytosol as a monomer that emits green fluorescence. The uptake of JC-1 into the mitochondria is dependent on MMP. Loss of MMP leads to increased green fluorescence in the cytosol and decreased red fluorescence in the mitochondria. Increased red fluorescence indicating aggregated mitochondrial JC-1 staining was observed in the α B-crystallin-overexpressing lens and retinal cells relative to control cells which showed decreased MMP under oxidative stress conditions (Fig. 4A). Even in the absence of oxidative stress treatment, the α B-crystallin/sHSP overexpressing cells showed increased red fluorescence relative to the control cells suggesting that α B-crystallin/sHSP may augment normal mitochondrial metabolism under non-oxidative conditions. As a control over-expression of α B-crystallin/sHSP was confirmed through analysis of α B-crystallin/sHSP transcript levels by RT-PCR of total RNA (Fig. 4B).

3.5 α B-crystallin/sHSP interacts with cytochrome c *in vivo*

Since α B-crystallin/sHSP was localized to the mitochondria (Figs. 1 and 2) and translocates to the mitochondria under stress conditions (Fig. 3), and since its over-expression of α B-crystallin preserved MMP under oxidative stress conditions in the lens and RPE cells (Fig. 4), we hypothesized that α B-crystallin/sHSP might preserve MMP at least in part through an ability to interact with and thereby protect one or more mitochondrial proteins against oxidative damage. As a potential target for the action of α B-crystallin/sHSP, we chose to examine cyt c since it is present at very high levels in the mitochondria and its oxidation at met-80 has been shown to directly decrease MMP and initiate apoptosis in HeLa cells [75]. To determine whether α B-crystallin/sHSP interacted with cyt c in the mitochondria of lens and retinal cells, we used fluorescent resonance energy transfer (FRET) analysis of cyt c-GFP and α B-dsRED transfected lens and RPE cells (Fig. 5). Excitation at the optimum wavelength for the donor (cyt c-GFP), 488 nm, led to donor quenching by the acceptor (α B-dsRED) and emission in the dsRED channel (red) indicating positive FRET and demonstrating interaction between cyt c-GFP and α B-dsRED in the lens and retinal cells (Fig. 5A). Control experiments using lens and RPE cells transfected with cyt c-GFP alone (Fig. 5B) and α B-dsRED alone (Fig. 5C) showed no positive emission and therefore no interaction. As a positive control, a GFP-dsRED fusion expressed in the lens and RPE cells showed a strong signal (Fig. 5D). To further confirm interaction between α B-crystallin/sHSP and cyt c co-immunoprecipitations of lens and RPE total protein extracts were performed. Western blot analysis revealed that α B-crystallin/sHSP co-immunoprecipitates with cyt c (Fig. 5E) using either an α B-crystallin-specific or a cyt c-specific antibody. Dimer forms of cyt c were detected in the immunoprecipitations as previously described for cyt c westerns of cellular protein extracts [62]. In addition to the major (20 kDa) α B-crystallin/sHSP band, a second lower molecular weight, α B-crystallin/sHSP immunoreactive band is apparent (Fig. 5E). Although, we have not confirmed the identity of this

band, we believe it to represent a proteolytic product of intact α B-crystallin/sHSP that reacts with the antibody.

3.6 α B-crystallin/sHSP protects cytochrome c against oxidative damage

Localization of α B-crystallin/sHSP to lens and retinal cell mitochondria (Fig. 1) coupled with its *in vivo* association with cyt c (Fig. 5) suggests that α B-crystallin/sHSP could confer mitochondrial protection at least in part by preventing oxidation and inactivation of cyt c. Previous studies demonstrated that cyt c is inactivated upon met-80 oxidation which causes disruption of iron binding to the protein and results in loss of cyt c electron transport, conformational changes and, ultimately, dissociation from the mitochondria and apoptotic induction [62, 63, 75].

The specific oxidation of cyt c met-80 to met-80 sulfoxide by HOCl-treatment [62, 63] causes a specific decrease in cyt c absorbance at 695 nm [63]. To evaluate the ability of α B-crystallin/sHSP to protect cyt c against oxidation, cyt c was pre-incubated with α B-crystallin/sHSP prior to exposure to HOCl, and the absorbance of cyt c at 695 nm was monitored relative to untreated cyt c and cyt c treated with HOCl in the absence of α B-crystallin/sHSP pre-incubation (Fig. 6A). Without HOCl treatment, cyt c exhibited a significant absorbance peak at 695 nm as previously described [63]. In the absence of α B-crystallin/sHSP pre-incubation, but with HOCl-treatment, the absorbance peak of cyt c at 695nm decreased to 42% ($p < 0.01$, $n = 3$) of the absorbance at 695 nm of untreated cyt c indicating oxidation of cyt c met-80 to met-80 sulfoxide upon HOCl-treatment. Pre-incubation of cyt c with α B-crystallin/sHSP at a 1:1 w/w ratio prior to subsequent HOCl-treatment yielded an absorbance of 76% ($p < 0.01$, $n = 3$) that of untreated cyt without α B-crystallin/sHSP suggesting that α B-crystallin/sHSP significantly protects cyt c against met-80 oxidation by HOCl.

Oxidation of met-80 in cyt c also activates peroxidase activity, providing an indirect measure of met-80 oxidation [62, 76]. To further examine if α B-crystallin/sHSP can protect cyt c against met-80 oxidation and thereby prevent cyt c inactivation, which also causes activation of its peroxidase activity we examined the peroxidase activity of cyt c exposed to oxidative conditions in the presence and absence of α B-crystallin/sHSP pre-incubation. Oxidation of cyt c with HOCl resulted in increased peroxidase activity compared to untreated cyt c (Fig. 6C) suggestive of met-80 oxidation as previously demonstrated [62, 76]. Pre-incubation of cyt c with α B-crystallin/sHSP in a 1:1 w/w ratio prior to HOCl-treatment resulted in significantly less peroxidase activity (54%, $p < 0.001$, $n = 3$) compared to cyt c oxidized in the absence of α B-crystallin/sHSP providing further evidence for direct oxidative protection of cyt c by α B-crystallin/sHSP. Protection of cyt c by α B-crystallin/sHSP appears to be specific for α B-crystallin/sHSP since little or no cyt c protection was observed when equal molar amounts of BSA were substituted for α B-crystallin/sHSP and cyt c peroxidase activity monitored under identical conditions (data not shown).

4. Discussion

In the present study we provide evidence that α B-crystallin/sHSP acts in lens and RPE cells to preserve mitochondrial function upon oxidative insult and that preservation of mitochondrial function by α B-crystallin/sHSP occurs at least in part through its ability to protect cyt c against oxidation at met-80. α B-crystallin/sHSP was localized to the mitochondria of lens and RPE cells by confocal microscopy using an antibody specific for α B-crystallin/sHSP (Fig. 1A) and by co-localization of dsRED tagged α B-crystallin/sHSP and the specific mitochondrial stain, Mitoview Green (Fig. 2). This data was further confirmed by western analysis of mitochondrial extracts (Fig. 1B). Demonstration of mitochondrial translocation in lens and retinal cells upon oxidative stress treatment (Fig. 3)

provides evidence for a specific α B-crystallin/sHSP function in lens and RPE cells under oxidative stress conditions. FRET imaging demonstrated that α B-crystallin/sHSP interacts with mitochondrial cytochrome c *in vivo* (Fig. 5). Although we cannot rule out the possibility that α B-crystallin/sHSP binds to the periphery of the mitochondrial surface rather than inside the mitochondria on the basis of confocal microscopy alone, we do not think that peripheral mitochondrial localization of α B-crystallin/sHSP in lens and RPE cells is likely since the FRET data showed a direct interaction of α B-crystallin/sHSP with cytochrome c and cytochrome c is located in the mitochondrial intermembrane/intercristae spaces under the non-apoptotic conditions examined [77]. Further electron microscopic studies will be necessary to pinpoint the exact location of α B-crystallin/sHSP in lens and RPE mitochondria.

The present data provide strong evidence that α B-crystallin/sHSP is localized to lens epithelial cell and RPE cell mitochondria where it likely functions to protect cytochrome c and other mitochondrial proteins against oxidative stress induced damage. The present data demonstrate that overexpression of α B-crystallin/sHSP in lens and RPE cells can preserve mitochondrial function under oxidative conditions by maintaining MMP (Fig. 4). Although MMP is only one of many parameters of mitochondrial function, this result nevertheless suggests that the electron transport function of mitochondria is preserved under oxidative stress conditions by the presence of α B-crystallin/sHSP. Consequently, it was also demonstrated that α B-crystallin/sHSP can protect cytochrome c against methionine oxidation *in vitro* (Fig. 6).

These data are consistent with recent studies demonstrating that preservation of mitochondrial function is essential for the viability of lens [66, 67] and RPE cells [68]. They are also consistent with a recent study demonstrating that the anti-oxidant enzyme MsrA can directly repair cytochrome c oxidized at methionine-80 and restore its electron transport function [62]. Interestingly, MsrA has also been shown to repair and restore the chaperone activity of total α -crystallin/sHSP damaged upon methionine oxidation [78]. Collectively, the data in the present report combined with these previous studies suggests that maintenance of lens and RPE cell mitochondria under oxidative stress is dependent upon a complex of proteins that consists at least in part of α B-crystallin/sHSP and MsrA.

The present data are also consistent with previous studies that demonstrate a major role for α B-crystallin/sHSP in the prevention of apoptosis in multiple cell types. Morrison *et al.*, observed that α B-crystallin/sHSP can inhibit caspase 3 activation and protect cardiomyocytes against hyperosmotic or hypoxic stress [35]. It was also observed that α B-crystallin/sHSP negatively regulates apoptosis during myogenesis by inhibiting the proteolytic activation of caspase-3 [36]. Liu *et al.*, found that expression of α B-crystallin/sHSP also prevents UVA-induced activation of the RAF/MEK/ERK pathway and abrogates UVA-induced apoptosis in human lens cells [38]. α B-crystallin/sHSP expression inhibits TRAIL (TNF-related apoptosis-inducing ligand)-induced apoptosis in nude mice. α B-crystallin/sHSP also prevents oxidative stress induced apoptosis by inhibiting caspase-3 activation in astrocytes [79]. It was found that both α A-crystallin/sHSP and α B-crystallin/sHSP bind to Bax and Bcl-XS *in vitro* and *in vivo* which could preserve mitochondrial integrity, inhibit cytochrome c release, and repress caspase-3 activation and PARP (Poly ADP-ribose polymerase) cleavage [37]. Since oxidation of cytochrome c is an initiator of the apoptotic pathway, our data also suggest that α B-crystallin/sHSP operates to control multiple steps important for the prevention of apoptosis including protection of cytochrome c against oxidation and subsequent prevention of cytochrome c release from the mitochondria.

In summary the present data provide evidence that α B-crystallin/sHSP protects lens and RPE cells against oxidative stress at least in part by preserving mitochondrial function in lens and retinal cells through its ability to interact with and prevent the oxidation of cytochrome c. In

addition to cyt c it is likely that α B-crystallin/sHSP interacts with and protects multiple mitochondrial proteins including MsrA. Since MsrA is a repair enzyme for cyt c oxidized at met-80 that can restore its electron transport function [62], it is possible that α B-crystallin/sHSP, MsrA and cyt c form a repair/protective complex that ensures cyt c functions in the mitochondria under stress conditions. It is likely that cyt c is but one of many mitochondrial targets for α B-crystallin/sHSP protection. α B-crystallin/sHSP is also localized to a multitude of other cellular components whose functions are also likely to be preserved by α B-crystallin/sHSP under oxidative stress conditions. The present data provide a window into at least one mechanism by which α B-crystallin/sHSP protects lens and retinal cells against oxidative stress damage that is applicable towards our understanding of its mode of action in other tissues and may shed light on its role in lens cataract, retinal degeneration, and other diseases.

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Abbreviations

Cyt c	cytochrome c
FRET	fluorescent resonance energy transfer
GFP	green fluorescent protein
HLE	human lens epithelial
Met-80	methionine-80
MMP	mitochondrial membrane potential
MsrA	methionine sulfoxide reductase A
PARP	poly ADP-ribose polymerase
RPE	retinal pigment epithelial
sHSP	small heat shock protein
TRAIL	TNF-related apoptosis-inducing ligand

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Highlights

Increased α B-crystallin/sHSP levels occur in oxidative stress diseases.

α B-crystallin/sHSP protects mitochondria and cyt c against oxidative inactivation.

Mitochondrial protection by α B-crystallin/sHSP likely prevents oxidative stress diseases.

Fig. 1. α B-crystallin/sHSP localizes to the mitochondria of lens and RPE cells
(A) Confocal microscopic images showing that α B-crystallin/sHSP (green) localizes to mitochondria (red) in lens and RPE cells and merging of the two images (yellow/orange). Scale bar: 20 μ m. **(B)** SDS-PAGE and immunoblot of whole cell, cytosolic and mitochondrial fractions from lens and RPE cells using an α B-crystallin-specific antibody, 5 μ g of total protein was loaded.

Fig. 2. dsRED-tagged αB-crystallin and GFP-tagged cytochrome c localize to the mitochondria of lens and RPE cells

(A) Confocal microscopic images showing lens and RPE cells transiently expressing αB-crystallin-dsRED (red), localized to the mitochondria stained with Mitoview Green (green), and merging of the two images (orange). Scale bar: 20 μm. (B) Confocal microscopic images showing lens and RPE cells transiently expressing cytochrome c-GFP (green), localized to the mitochondria stained with mitochondrial stain Mitotracker Red (red), and merging of the two images (yellow/orange). Scale bar: 20 μm.

Fig. 3. αB-crystallin/sHSP translocates to the mitochondria of lens and RPE cells under oxidative stress exposure

(A) SDS-PAGE and immunoblot of cytosolic and mitochondrial fractions from lens cells treated with 0 μM, 100 μM or 200 μM H₂O₂ for 1 h. Immunoblots were probed with an αB-crystallin-specific antibody, 10 μg of total protein was loaded. Colloidal blue staining is shown as a control for equal protein loading. (B) SDS-PAGE and immunoblot of cytosolic and mitochondrial fractions from RPE cells treated with 0 μM, 100 μM or 200 μM H₂O₂ for 1 h. An αB-crystallin-specific antibody was used and 10 μg of total protein was loaded. Colloidal blue staining is shown as a control for equal protein loading.

Fig. 4. α B-crystallin/sHSP protects mitochondrial membrane potential of lens and RPE cells under oxidative stress conditions

(A) Confocal microscopic images of control lens and RPE cells and α B-crystallin/sHSP overexpressing (α B L/V) lens and RPE cells treated with 0 μ M and 50 μ M H_2O_2 for 24 h. Cells were stained with JC-1 for 20 min to detect changes in mitochondrial membrane potential as indicated by red mitochondrial staining (increased potential) or green cytosolic staining (decreased potential). This is reflected in the merged image as increased yellow fluorescence indicating decreased MMP. Scale bar: 20 μ m. (B) Ethidium bromide stained gels showing levels of α B-crystallin and control GAPDH transcript in 200 ng RNA isolated from lens and RPE control cell lines and lens and RPE cells overexpressing α B-crystallin/sHSP (α B L/V).

Fig. 5. αB-crystallin/HSP interacts with cytochrome c *in vivo*

(A) FRET analysis was performed on lens and RPE cells expressing αB-dsRED and/or cyt c-GFP. Cells were excited with a 488nm laser and FRET emissions detected in the dsRED channel. (B) Donor only (Cyt c-GFP) and (C) acceptor only (αB-dsRED) were used as negative FRET controls and (D) dsRED-GFP fusion protein served as a positive control. Scale bar: 20 μm. (E) Co-immunoprecipitations of lens and RPE whole cell lysate using an αB-crystallin-specific antibody (αB-IP) or a cyt c-specific antibody (cyt c-IP) and probed with antibodies specific for cyt c or αB-crystallin.

Fig. 6. αB-crystallin/sHSP preserves cytochrome c function under oxidative stress conditions
(A) Visible absorption spectra of cyt c. Oxidation of cyt c met-80 by HOCl shown by the disappearance of the cyt c 695 nm peak. Absorption spectra were measured before and after treatment with HOCl at a 12:1 molar ratio for 15 min with and without αB-crystallin/sHSP pre-incubation. **(B)** Absorption of cyt c at 695 nm. Absorbance of cyt c without HOCl treatment was assigned with a value of 100%. Cyt c was oxidized at 12:1 (molar ratio) with HOCl for 15 min with and without pre-incubation with αB-crystallin/sHSP. Absorptions are shown as a percentage of the absorbance of untreated cyt c. p values were obtained using the students t-test (n=3) **(C)** Representative graph of cyt c peroxidase activity. Oxidized cyt c (incubated for 15 min with a 12:1 molar ratio of HOCl:cyt c) gives maximal peroxidase activity (100%). Pre-incubation of cyt c with αB-crystallin/sHSP before oxidation (at 1:1 w/w ratio) leads to a decrease in peroxidase activity following oxidation. Activities are shown here as a percentage of the activity of the oxidized form of cyt c. p values were obtained using the students t-test (n=3).

Table 1

The forward and reverse primers for subcloning experiments of α B-crystallin into pDsRED-monomer-c1, cyt c into pAcGFP-C1 and RED into pAcGFP-C1 vectors. The underlined sequences indicate the restriction sites for each.

Insert	Forward primer		Reverse primer	
α B-crystallin	AACCCTCGAGCTGACATC GCCATCCAC	XhoI	CAGGAGGAATTCCTATTTCT TGGGGGC	EcoRI
Cyt c	CTCAGATCTCGAGCTCAA GCTTCGATGGGTGATGTT GAGAAA	HindIII	GCAATTCTAGATTATAGTAG CTTTTTGAGATAAGCT	XbaI
dsRED	AAAGGTACCCCGGTCGCC ACCATGGACA	KpnI	TTTGGATCCCTGGGAGCCG GAG	BamHI