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Interactive sequences in the stress protein and molecular chaperone human α B crystallin recognize and modulate the assembly of filaments

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Abstract

Molecular chaperones including the small heat shock protein, α B crystallin and sHSP27 participate in the assembly, disassembly, and reorganization of the cytoskeleton during cell development and differentiation. While α B crystallin and sHSP27 stabilize and modulate filament assembly and reorganization, the sequences and structural domains mediating interactions between these proteins and filaments are unknown. It is important to define these interactive domains in order to understand differential interactions between chaperones and stable or unfolding filaments and their function in the cellular stress response. Protein pin arrays identified sequences in human α B crystallin that selectively interacted with native or partially unfolded filament proteins desmin, glial-fibrillary acidic protein, and actin. Circular dichroism spectroscopy determined the differences in structure of these filaments at 23°C and 45°C. Seven α B crystallin sequences had stronger interactions with desmin and six sequences had stronger interactions with glial-fibrillary acidic protein at 23°C than at 45°C. The α B crystalline sequences ₃₃LESDFPTSTSLSPFYLRPPSFLR₅₆ and ₁₂₉DPLTITSSLSSDGV₁₄₅ had the strongest interactions with actin at 23°C, while ₅₇APSWFDTG₆₄, ₁₁₁HGFISREF₁₁₈, ₁₄₅VNGPRKQVSG₁₅₄, and ₁₅₅PERTIPITREEK₁₆₅ had the strongest interactions with actin at 45°C. The actin interactive sequences of α B crystallin overlapped with previously identified α B crystallin chaperone sequences and were synthesized to evaluate their effect on the assembly and aggregation of actin. Full-length α B crystallin and the core domain chaperone sequence ₁₃₁LTITSSLSSDGV₁₄₃ promoted actin polymerization at 37°C and inhibited depolymerization and aggregation at 50°C. The results support the hypothesis that interactive domains in α B crystallin have multiple functions in stabilizing the cytoskeleton and protecting cytosolic proteins from unfolding.

Keywords

molecular chaperone; small heat shock protein; α crystallin; actin; intermediate filament; desmin-related myopathy (DRM); glial-fibrillary acidic protein

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Introduction

Interactions between small heat shock proteins (sHSPs) and filaments are important during normal cell differentiation and in protein aggregation diseases (Iwaki et al., 1989; Kato et al., 1992; Prescott et al., 1996; van Rijk and Bloemendal, 2000). An association between α crystallin, the archetype of sHSPs, and the cytoskeletal network has been recognized since the earliest observations of beaded filaments in extracted lens cells (Bagchi, Katar, & Maisel, 2002; Carter, Hutcheson, & Quinlan, 1995; FitzGerald & Graham, 1991; Ireland & Maisel, 1989; R. A. Quinlan, Carte, Sandilands, & Prescott, 1996). The lens fiber cytoplasm contains a dense network of filaments, which are required for normal transparency and lens refraction and the lens cytoskeleton is intimately associated with α crystallin (Liang & MacRae, 1997; MacRae, 2000; Nicholl & Quinlan, 1994; Perng, Cairns et al., 1999; Sandilands et al., 1995). It is believed that sHSPs participate in the dynamic assembly and stabilization of the filamentous cytoskeleton (Melkani, Cammarato, & Bernstein, 2006; Singh, Rao, Ramakrishna, Rangaraj, & Rao, 2006). In normal tissues, α B crystallin interacts directly with a number of filament proteins including phakinin (CP49), filensin (CP119), desmin, glial fibrillary acidic protein (GFAP), vimentin, and actin where it functions in the organization and stabilization of the filament networks formed by these proteins (Bennardini, Wrzosek, & Chiesi, 1992; Djabali, Piron, de Nechaud, & Portier, 1999; Gopalakrishnan, Boyle, & Takemoto, 1993; Head, Hurwitz, Kegel, & Goldman, 2000; Launay, Goudeau, Kato, Vicart, & Lilienbaum, 2006; Liang & MacRae, 1997; Lieska, Chen, Maisel, & Romero-Herrera, 1980; Maglara, Vasilaki, Jackson, & McArdle, 2003; Muchowski, Valdez, & Clark, 1999; Prescott, Sandilands, Hutcheson, Carter, & Quinlan, 1996; R. Quinlan, 2002; Tomokane, Iwaki, Tateishi, Iwaki, & Goldman, 1991; Wisniewski & Goldman, 1998). Mutations in α B crystallin including the R120G mutation that alters the selective association between α B crystallin and filaments cause cataracts, myofibrillar myopathy and desmin-related cardiomyopathy (DRM) apparently through the defective assembly of intracellular filament networks (Bova et al., 1999; Litt et al., 1998; Selcen & Engel, 2003; Vicart et al., 1998).

Identification and characterization of the filament interactive sites in human α B crystallin will provide a structural basis for understanding the function of sHSPs in the recognition, solubilization, assembly, and stabilization of filament networks. In the current study, human α B crystallin sequences that interacted with cytoskeletal filaments in their native or partially unfolded states were identified using protein pin arrays that were used previously to identify interactive sequences for chaperone activity and subunit-subunit interactions in α B crystallin (Ghosh & Clark, 2005; Ghosh, Estrada, & Clark, 2005). The α B crystallin filament interactive sequences identified by the pin arrays selectively recognized and interacted with partially unfolded desmin and GFAP, and native and partially unfolded actin. In addition, these sequences overlapped with previously identified subunit-subunit and chaperone sequences of α B crystallin suggesting a novel mechanism for sHSP functions *in vivo* that depends on the selective recognition of the unfolding state of filament and chaperone target proteins.

Materials and Methods

Synthesis of α B crystallin peptides

The α B crystallin peptides $_{41}\text{STSLSPFYLRPPSFLRAP}_{58}$ (ST), $_{113}\text{FISREFHR}_{120}$ (FI), $_{131}\text{LTITSSLSDDGV}_{142}$ (LT), and $_{156}\text{ERTIPITRE}_{164}$ (ER) were synthesized by Genscript Corporation (Piscataway, NJ).

Ultra-violet circular dichroism spectroscopy

The secondary and tertiary structure of each filament protein was determined using ultra-violet circular dichroism (UVCD) at 23°C and after heating at 45°C for 15mins. A concentration of

0.02mg/ml protein was used for far UVCD measurements (200-249nm) and a concentration of 0.1mg/ml was used for near UVCD measurements (250-320nm). The UVCD spectrum of each protein was adjusted to the baseline using the UVCD spectrum of PBS buffer (5mM phosphate buffer, 1.3mM potassium chloride and 137mM sodium chloride, pH 7.0). The far UVCD spectra of the filament proteins were deconvoluted using Dichroweb (Lobley, Whitmore, & Wallace, 2002; Whitmore & Wallace, 2004) to calculate the secondary structure composition of the filaments.

Protein pin array assay

A protein pin array (Mimotopes, San Diego, CA) consisting of eighty-four eight amino acid long peptides corresponding to the primary sequence of human α B crystallin was synthesized and used to measure interactions with desmin, glial fibrillary acid protein (GFAP), and actin as described previously (Geysen, 1990; Ghosh & Clark, 2005). Proteins and antibodies purchased from suppliers are listed in Table 1. The purity of the filament proteins, desmin, GFAP and actin used in the pin array assays were determined to be >95% by SDS-PAGE analysis (data not shown). Primary antibodies used in the pin array assays were specific to each target protein and minor contaminating proteins that bound to the α B crystallin peptides were not detected. Positive interactions resulted in blue color and were plotted as absorbance at $\lambda = 450\text{nm}$ and plotted on the Y-axis. The sequences of the α B crystallin peptides were plotted on the X-axis. The pin array assays were conducted in duplicate with filament proteins at 23°C or with proteins heated at 45°C for 15mins. Human myoglobin did not interact with any of the α B crystallin peptides and was the negative control for the protein pin array assay (Ghosh & Clark, 2005). A single pin array was used for all experiments and no significant decrease in efficiency was observed even after repeated use up to thirty times. The last three peptides of the protein pin array correspond to the epitope, $_{163}\text{REEKPAVTAAPKK}_{175}$, recognized by the primary antibody for α B crystallin and were used to measure the efficiency of the pin array as described previously (Ghosh, Estrada, & Clark, 2005). The loss of efficiency for the pin array was determined to be <5% by after more than thirty assays. Average absorbances were calculated by averaging the measured absorbances of all eighty-four α B crystallin peptides at each temperature. The percentage change in absorbance was calculated as follows:

$$\text{Percent Change} = \frac{\text{Average absorbance at } 45^{\circ}\text{C}}{\text{Average absorbance at } 23^{\circ}\text{C}}$$

Actin polymerization and thermal aggregation assay

The effects of the α B crystallin peptides that had strong interactions with actin in the pin arrays on actin polymerization were evaluated using an Actin Polymerization Kit using the protocol supplied by the manufacturer (Cytoskeleton; Denver, CO)(Kumar, Tomar, Parrill, & Khurana, 2004). Polymerization of globular actin (G-actin) to filamentous actin (F-actin) results in incorporation of pyrene which increases pyrene fluorescence at $\lambda = 460\text{nm}$. 4.5ml of 5mM Tris-HCl, 0.2mM CaCl_2 , 0.2mM ATP, pH 8.0 was mixed with 100 μl of 10mg/ml pyrene-labeled actin and incubated at 25°C for 60mins. 80 μl of the sample was added to a well containing 8 μl of 250 μM peptide in 0.31% DMSO. Fluorescence of the samples were recorded at room temperature for 20mins prior to the addition of Mg^{++} using a Victor3 V fluorescence plate reader (Excitation $\lambda = 355\text{nm}$, Emission $\lambda = 460\text{nm}$). 10 μl of 50mM KCl, 2mM MgCl_2 , 1mM ATP was then added to each sample and fluorescence was recorded continuously for 125mins. Subsequently, the plate was heated at 50°C to initiate thermal aggregation and the fluorescence was recorded for 60mins to evaluate the effect of the α B crystallin peptides on the aggregation of F-actin.

Note: Previous studies reported the critical concentrations for the assembly of desmin and GFAP filaments were $\sim 0.08\mu\text{g/ml}$ and $\sim 0.12\mu\text{g/ml}$ respectively (Chou, Stromer, Robson, & Huiatt, 1990; Orban, Halasi, Papp, Barko, & Bugyi, 2005; Pan & Ware, 1988; Papp, Bugyi, Ujfalusi, Halasi, & Orban, 2005; F. Wang, Sampogna, & Ware, 1989; Yang & Babitch, 1988). UVCD measurements were conducted at protein concentrations of 2mg/ml , while pin array assays were conducted at protein concentrations of $25\mu\text{g/ml}$, both of which are well above the critical concentration for filament assembly.

Results

The secondary and tertiary structure of the desmin, GFAP, and actin were determined at 23°C and 45°C by UVCD spectroscopy (Fig. 1 and Table 2). The deconvoluted far UVCD spectrum for desmin at 23°C corresponded to 16% helix, 47% coil, and 37% β sheet secondary structure. When desmin was heated at 45°C for 15mins, the helical content in the secondary structure of desmin increased from 16% to 80% helix, random coil decreased from 47% to 19% coil, and β sheet decreased from 37% to 1%. The deconvoluted far UVCD spectrum of GFAP at 23°C corresponded to a secondary structure of 28% helix, 53% coil, and 19% β sheet. At 45°C for 15mins, the helical content of GFAP increased from 28% to 40% helix, coil decreased from 53% to 44% and β sheet decreased from 19% to 16%. The deconvoluted far UVCD spectrum of actin at 23°C corresponded to a secondary structure of 23% helix, 52% coil, and 25% β sheet. At 45°C , there was a small decrease in helical content from 23% to 18%, a decrease from 52% coil to 48% and an increase from 25% to 34% β sheet. Desmin and GFAP had higher helical content at 45°C than at 23°C , while actin had slightly lower helical content at 45°C than at 23°C .

Interactions between the intermediate filament protein desmin and the eighty-four αB crystallin peptides were measured using a protein pin array (Fig. 2 and Table 3). When the pin array assay was conducted with desmin at 23°C , absorbances were observed in the wells corresponding to eighty-three of the eighty-four peptides. The highest absorbances were observed for peptides corresponding to the N-terminal sequences $_{15}\text{FPFHSPSRLF}_{24}$ and $_{41}\text{STSLSPFYLRPPSFLRAPS WFD}_{62}$, the α crystalline core domain sequences, $_{75}\text{FSVNLDVKHFSPEELK}_{90}$, $_{113}\text{FISREFHRKYRIPADV}_{128}$, $_{131}\text{LTITSSLS}_{138}$, and $_{145}\text{VNGPRKQV}_{152}$, and the C-terminal sequence, $_{163}\text{REEKPAVTAAPK}_{174}$. When the pin array assay was conducted with desmin at 45°C , the magnitude of the absorbances of eighty-three αB crystallin peptides was approximately 45% of the magnitude at 23°C . The αB crystallin peptides had stronger interactions with desmin at 23°C than at 45°C the temperature corresponding to high helical content characteristic of structured intermediate filaments (Herrmann & Aebi, 2004).

Interactions between the intermediate filament protein GFAP and eighty-four αB crystallin peptides were measured using a protein pin array (Fig. 3 and Table 3). When the pin array assay was conducted with GFAP at 23°C , absorbances were observed in the wells corresponding to eighty-two of eighty-four αB crystallin sequences. Peptides that had the highest absorbances with GFAP at 23°C were $_{9}\text{WIRPFFP}_{16}$ and $_{45}\text{SPFYLRPPSFLRAPS WFD}_{62}$ in the αB crystallin N-terminus, $_{89}\text{LKV KVLGDVI}_{98}$, $_{113}\text{FISREFHRKYRIPADV}_{128}$, and $_{143}\text{LTVNGPRK}_{150}$ in the α crystallin core domain and, $_{155}\text{PERTIPITREEKPAVTAA}_{172}$ in the C-terminus. When the pin array assay was conducted with GFAP at 45°C , the magnitude of the absorbances of was approximately 23% of the magnitude at 23°C . While 82/84 αB crystallin peptides are observed to interact with GFAP at 23°C , only 33/84 peptides had signals stronger than the baseline value of 0.1. At 45°C , 40/84 αB crystallin peptides interacted with GFAP and only 22/84 peptides had signals greater than 0.1. The αB crystallin peptides had stronger interactions with GFAP

at 23°C than at 45°C the temperature corresponding to high helical content characteristic of structured intermediate filaments (Herrmann & Aebi, 2004).

Interactions between the cytoskeletal microfilament protein actin and eighty-four α B crystallin peptides were measured using a protein pin array (Fig. 4 and Table 3). When the pin array assay was conducted with actin at 23°C, absorbances were observed in the wells corresponding to all eighty-four α B crystallin sequences. When the pin array assay was conducted with actin at 45°C, the magnitude of absorbances of the α B crystallin peptides was approximately 91% of the magnitude at 23°C. Peptides corresponding to the N-terminal sequence $_{33}\text{LESDFPTSTSLSPFYLRPPSFLR}_{56}$ and the C-terminal sequence $_{129}\text{DPLTITSSLSSDGV}_{142}$ had the highest absorbances with actin at 23°C, while peptides corresponding to the N-terminal sequences $_{15}\text{FPFHSPSRLFDQ}_{26}$ and $_{57}\text{APSWFDTG}_{64}$, the α crystallin core domain sequence $_{111}\text{HGFISREF}_{118}$, and the C-terminal sequences $_{145}\text{VNGPRKQVSG}_{154}$ and $_{155}\text{PERTIPITREEK}_{165}$ had the highest absorbances with actin at 45°C.

The four α B crystallin sequences $_{41}\text{STSLSPFYLRPPSFLRAP}_{58}$, $_{113}\text{FISREFHF}_{120}$, $_{131}\text{LTITSSLSSDGV}_{142}$, and $_{156}\text{ERTIPITRE}_{164}$ that had strong interactions with actin in the pin array assay overlapped with previously identified chaperone sequences of α B crystallin (Ghosh, Estrada, & Clark, 2005, 2006; Ghosh, Estrada, Houck, & Clark, 2006). Consequently, these four peptides were synthesized to determine their individual effects on the polymerization and thermal aggregation of unassembled globular actin (G-actin) and assembled filamentous actin (F-actin) using pyrene-labeled actin (Fig. 5). When 4 μ M pyrene-labeled actin alone was incubated in the polymerization buffer at 37°C, the pyrene fluorescence at $\lambda = 460\text{nm}$ increased to a maximum value of 8187 RFU after 125mins. The increased fluorescence was due to incorporation of pyrene-labeled actin into assembled actin filaments. In contrast, incubation of 4 μ M pyrene-labeled actin at 37°C in the absence of polymerization buffer resulted in a decrease in fluorescence at $\lambda = 460\text{nm}$ to a minimum value of -8792 RFU due to quenching of pyrene-fluorescence by the solvent. When 4 μ M pyrene-labeled actin was incubated with 4 μ M full-length wt human α B crystallin, the fluorescence at $\lambda = 460\text{nm}$ increased rapidly and reached a maximum value of 19276 RFU. When 4 μ M pyrene-labeled actin was incubated with 20 μ M of the synthetic peptides ST and ER in the polymerization buffer at 37°C, the fluorescence at $\lambda = 460\text{nm}$ increased rapidly and reached maximum values of 9533 RFU and 10,000 RFU respectively in approximately 125mins. The fluorescence maxima of the samples containing the ST and ER synthetic peptides were not significantly different from the sample containing actin alone. In contrast, incubation of 4 μ M pyrene-labeled actin with 20 μ M FI increased the fluorescence at $\lambda = 460\text{nm}$ to a maximum of 12407 RFU without an effect on the rate of polymerization of G-actin to F-actin. Incubation of 4 μ M pyrene-labeled actin with 20 μ M LT increased the rate of polymerization and the fluorescence maximum to 12790 RFU after 125mins. Full-length wt human α B crystallin and the synthetic peptides FI and LT had a positive effect on actin polymerization, while the ST and ER synthetic peptides had little to no effect on actin polymerization.

After complete polymerization was achieved at 125mins, the samples were heated to 50°C for 60mins to evaluate the effect of the synthetic peptides on the thermal stability of F-actin. After 60mins of incubation at 50°C, the fluorescence of unpolymerized G-actin incubated without polymerization buffer increased dramatically from -8792 to 7835 RFU indicating thermal aggregation of G-actin. Similarly, after 60mins of incubation at 50°C, the fluorescence of F-actin increased from -8187 to 14370 RFU indicating thermal aggregation of F-actin. Full-length wt human α B crystallin protected against the thermal aggregation of F-actin and the fluorescence of the sample containing full-length wt human α B crystallin decreased from 19276 to 13308 RFU after 60mins at 50°C. The α B crystallin peptides ST, FI and ER had no

significant effect on the thermal stability of F-actin and the fluorescence of all three samples increased after heating at 50°C for 60mins. In contrast, the fluorescence of the sample containing the LT peptide decreased from 12790 RFU at 37°C to 10247 RFU after heating at 50°C for 60mins indicating protection against the thermal aggregation of F-actin. Full-length wt human α B crystallin and the chaperone peptide LT protected against the thermal aggregation of actin, while the ST, FI and ER synthetic peptides had no effect on the thermal aggregation of actin.

Discussion

Protein pin arrays identified sequences in α B crystallin used for interactions with the intermediate filaments desmin and GFAP, and the microfilament actin. The results were consistent with previous reports of interaction between α B crystallin and the filaments desmin, GFAP, and actin (Launay, Goudeau, Kato, Vicart, & Lilienbaum, 2006; Muchowski, Valdez, & Clark, 1999; Nicholl & Quinlan, 1994; Perng, Cairns et al., 1999; R. Quinlan, 2002; R. A. Quinlan, Carte, Sandilands, & Prescott, 1996). Pin array analysis of the α B crystallin-desmin interactions determined that the α B crystallin peptide $_{113}\text{FISREFHR}_{120}$ had strong interactions with human desmin. The result confirmed the importance of the R120G mutation in human α B crystallin which is responsible for abnormal interactions between human α B crystallin and desmin filaments leading to cataracts in the filament cardiomyopathy, desmin-related myopathy (DRM) (Bova et al., 1999; Inagaki et al., 2006; Litt et al., 1998; Perng, Muchowski et al., 1999; Perng, Wen, van den, Prescott, & Quinlan, 2004; Selcen & Engel, 2003; Sun & Macrae, 2005; Vicart et al., 1998). Similarly, pin array analysis determined that the sequence, $_{57}\text{APSWFDTG}_{64}$ had strong interactions with partially unfolded actin. This interactive sequence contains Ser-59, a key residue in human α B crystallin that modulates interactions with actin filaments in response to cardiac stress (Ecroyd et al., 2006; Kato et al., 2001; Morrison, Hoover, Thuerlauf, & Glembotski, 2003; Shu et al., 2005; Webster, 2003). Phosphorylation of Ser-59 results in increased interactions between α B crystallin and actin that is believed to protect actin filaments from unfolding and aggregation (Launay, Goudeau, Kato, Vicart, & Lilienbaum, 2006). The result confirmed the importance of the Ser-59 residue in interactions between α B crystallin and actin filaments under stress.

Synthesis of the individual α B crystallin peptides permitted an evaluation of the function of each interactive domain on actin polymerization and aggregation. The α B crystallin core domain sequence $_{131}\text{LTITSSLSSDGV}_{142}$ that was previously shown to have chaperone activity *in vitro* (Ghosh & Clark, 2005) promoted the polymerization of G-actin into F-actin and protected against the aggregation of actin filaments. In contrast, the α crystallin core domain sequence $_{113}\text{FISREFHR}_{120}$ promoted actin polymerization without a measurable effect on the aggregation of F-actin. The N-terminal sequence $_{41}\text{STSLSPFYLRPPSFLRAP}_{58}$ and the C-terminal sequence $_{156}\text{ERTIPITRE}_{164}$ had no measurable effect on the polymerization and aggregation of actin, which was consistent with recent studies in which these two sequences were observed to have no intrinsic chaperone activity but were shown to play a critical role in the recognition, selection, and solubilization of substrate proteins (Ghosh, Shenoy, & Clark, 2006). The results suggested that the sequences $_{41}\text{STSLSPFYLRPPSFLRAP}_{58}$ and $_{156}\text{ERTIPITRE}_{164}$ facilitate the recognition and binding of native and partially unfolded actin by human α B crystallin under normal and stress conditions. The results support the hypothesis that under physiological conditions, α B crystallin facilitates the assembly of actin filaments, while under thermal and oxidative stress, α B crystallin protects actin filaments from unfolding and aggregation (Benndorf et al., 1994; Gopalakrishnan & Takemoto, 1992; Liang & MacRae, 1997; Longoni, Lattonen, Bullock, & Chiesi, 1990; Mounier & Arrigo, 2002; K. Wang & Spector, 1996). Pin array analysis, actin polymerization, and thermal aggregation assays indicated that α B crystallin employs common interactive sequences to promote the assembly of actin filaments and stabilize actin filaments.

The presence of common α B crystallin interactive sequences for cytoskeletal filament proteins and cytosolic lens crystallins (Table 3 and Fig. 6) suggested a novel mechanism for the chaperone activity of α B crystallin in filament assembly and chaperone activity. Differences in the UVCD spectra for desmin and GFAP at 23°C and 45°C determined that the filaments were partially unfolded and contained low helical secondary structure at 23°C but not at 45°C. In contrast, the cytosolic lens crystallins β/γ crystallins were stable at 23°C and begin unfolding at temperatures >45°C (Ghosh, Estrada, & Clark, 2005). The same α B crystallin sequences that had weak or no interactions with β/γ crystallins at 23°C (Ghosh, Estrada, & Clark, 2005) had strong interactions with desmin and GFAP at 23°C. The results suggest a preferential interaction with filaments relative to β/γ crystallins at 23°C and a preferential interaction with unfolding β/γ crystallins at 45°C relative to the filaments (Ghosh, Estrada, & Clark, 2005). In the absence of stress, α B crystallin interacts with and stabilizes filaments. Under conditions of stress, α B crystallin dissociates from the filaments to interact with unfolding β/γ crystallins using the same interactive surface domains to protect against aggregation and loss of transparency. The R120G mutation is an example of a post-translational modification that modifies the interactive sites of α B crystallin in a key surface domain to disrupt both α B crystallin-filament interactions and the α B crystallin response to unfolding proteins (Bloemendal et al., 2004; Fu & Liang, 2003; Perng, Wen, van den, Prescott, & Quinlan, 2004). The results are consistent with the hypothesis that filaments play an important role in the dynamic subunit model for chaperone activity of sHSPs (Launay, Goudeau, Kato, Vicart, & Lilienbaum, 2006; Liu, Ghosh, Clark, & Jiang, 2006).

In summary, pin arrays identified interactive domains on the molecular chaperone, human α B crystallin that mediated interactions with the filament proteins desmin, GFAP, and actin. The presence of common functional surface domains that mediate interactions with both filaments and cytosolic lens crystallins suggested that differential affinities between α B crystallin and substrate proteins may be the basis for the recognition, selection, and binding steps of sHSP chaperone function. Further experiments are underway using surface plasmon resonance to quantify the functional significance of differences in affinities between α B crystallin subunits, filaments and unfolded proteins in a mechanism involving dynamic subunit exchange in the unfolding protein response.

Acknowledgments

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Fig. 1.

UVCD spectroscopic analysis of the effect of heat on the structure of human desmin (A), human GFAP (B), and rabbit muscle actin (C). The far UVCD spectra of desmin, GFAP, and actin were measured at 23°C and after heating at 45°C for 15mins, which was identical to the conditions used in the pin array assays. In the desmin spectrum, the negative ellipticity at 222nm increased from $-4467 \text{ deg.cm}^2.\text{dmol}^{-1}$ at 23°C to $-24677 \text{ deg.cm}^2.\text{dmol}^{-1}$ at 45°C indicating a gain of helical secondary structure with temperature which was consistent with previous reports (Fuchs & Weber, 1994). In the GFAP spectrum, the negative ellipticity at 222nm increased from $-8596 \text{ deg.cm}^2.\text{dmol}^{-1}$ at 23°C to $-11230 \text{ deg.cm}^2.\text{dmol}^{-1}$ at 45°C indicating a gain in helical secondary structure with temperature which was consistent with previous reports (Fuchs & Weber, 1994). Actin was thermostable up to 45°C, and the negative ellipticity at 222nm decreased from $-2787 \text{ deg.cm}^2.\text{dmol}^{-1}$ at 23°C to $-2753 \text{ deg.cm}^2.\text{dmol}^{-1}$ at 45°C, which was consistent with previous reports (Bertazzon, Tian, Lamblin, & Tsong, 1990; Bertazzon & Tsong, 1990). Based on UVCD, desmin and GFAP were determined to have more helical content at 45°C than at 23°C, while actin had slightly lower helical content at 45°C than at 23°C.

Fig. 2.

Pattern of interaction between human α B crystallin peptides and human desmin. The amino acid sequence of each α B crystallin eight-mer peptide is listed on the X-axis. The Y-axis plots the absorbances measured at 450nm for the α B crystallin peptides in the presence of desmin at 23°C (clear bars) and 45°C (solid black bars). Grey bars represent the difference in the recorded absorbance of each peptide with desmin at 23°C and 45°C. Positive grey bars represent peptides that had a stronger interaction at 23°C than at 45°C. The heights of the bars are a measure of the strength of the interactions between α B crystallin peptides and desmin. Peptides that had the strongest interactions with desmin were flanked on either side by peptides that had weak or no interactions with desmin resulting in a pattern of high and low absorbances. All eighty-four α B crystallin peptides interacted with desmin at 23°C and eighty-three of eighty-four peptides interacted with desmin at 45°C. At 45°C, the average absorbance of the α B crystallin pin array was 45% of the average absorbance at 23°C. The seven α B crystallin sequences that had the strongest interactions were labeled and identified as desmin interactive sequences of human α B crystallin.

Fig. 3.

Pattern of interaction between human α B crystallin peptides and human GFAP. The amino acid sequence of each α B crystallin eight-mer peptide is listed on the X-axis. The Y-axis plots the absorbances measured at 450nm for the α B crystallin peptides in the presence of GFAP at 23°C (clear bars) and 45°C (solid black bars). Grey bars represent the difference in the recorded absorbance of each peptide at 23°C and 45°C. Positive grey bars represent peptides that had a stronger interaction with GFAP at 23°C than at 45°C. The heights of the bars are a measure of the strength of the interactions between α B crystallin peptides and GFAP. Eighty-two of eighty-four α B crystallin peptides interacted with GFAP at 23°C and thirty-seven of eighty-four peptides interacted with GFAP at 45°C. At 45°C, the average absorbance of the α B crystallin pin array was 23% of the average absorbance at 23°C. The six α B crystallin sequences that had the strongest interactions with unheated GFAP are labeled and identified as GFAP interactive sequences.

Fig. 4.

Pattern of interaction between human α B crystallin peptides and rabbit muscle actin. The amino acid sequence of each α B crystallin eight-mer peptide is listed on the X-axis. The Y-axis plots the absorbances measured at 450nm for the α B crystallin peptides in the presence of actin at 23°C (clear bars) and 45°C (solid black bars). Grey bars represent the difference in the recorded absorbance for each peptide at 23°C and 45°C. Positive grey bars represent peptides that had a stronger interaction with actin at 23°C than with actin at 45°C. Negative grey bars represent peptides that had stronger interactions with actin at 45°C than with actin at 23°C. Thirty-seven of eighty-four α B crystallin peptides had stronger interactions with actin at 23°C than with actin at 45°C (positive grey bars), thirty-four peptides had stronger interactions with actin at 45°C than actin at 23°C (negative grey bars), and thirteen peptides had similar interactions with both actin at 23°C and 45°C. The two α B crystallin sequences that had the strongest interactions with actin at 23°C, and the four α B crystallin sequences that had the strongest interactions with actin at 45°C are labeled and identified as actin interactive sequences of human α B crystallin. At 45°C, the average absorbance of the α B crystallin pin array was 91% of the average absorbance at 23°C.

Fig. 5.

Comparison of select α B crystallin peptides with full-length wt human α B crystallin on the polymerization and thermal aggregation of actin. In the presence of polymerization buffer (PB) only (solid grey line), G-actin polymerization to F-actin was measured as an increase in fluorescence at $\lambda = 460\text{nm}$ at 37°C in approximately 125mins. In the absence of polymerization buffer, no polymerization was observed and the fluorescence at $\lambda = 460\text{nm}$ decreased (thin broken line). Wt human α B crystallin (solid black line) increased both the rate and the total amount of polymerization of G-actin to F-actin. The synthetic α B crystallin peptides ST ($\square$ -) and ER ($\circ$ -) had no effect on the polymerization of G-actin to F-actin, while the FI peptide ($\diamond$ -) increased the amount of polymerization but had no effect on the rate of polymerization of G-actin to F-actin. The LT peptide (Δ -) increased both the rate and the total amount of polymerization of G-actin to F-actin similar to wt α B crystallin. When unpolymerized G-actin (thin broken line) was heated to 50°C for 60mins increased aggregation of G-actin was

measured as increased fluorescence at $\lambda = 460\text{nm}$. Similarly, the fluorescence at $\lambda = 460\text{nm}$ of the sample containing F-actin alone (solid grey line) increased upon heating at 50°C for 60mins indicating aggregation of F-actin. Wt human αB crystallin (solid black line) protected against the aggregation of F-actin at 50°C . No protection against thermal aggregation of F-actin was observed in the presence of the synthetic peptides ST ($-\square-$), FI ($-\diamond-$), and ER ($-\circ-$). In contrast, protection against thermal aggregation of F-actin was observed in the presence of the peptide LT ($-\Delta-$). The action of the peptide, LT, resembled the action of full-length wt human αB crystallin which was the strongest promoter of actin polymerization and the strongest inhibitor of F-actin aggregation. Errors bars are shown for only the actin-alone sample to prevent overcrowding of the figure. The data shown are an average of three experiments performed in triplicate.

Fig. 6. Interactive surfaces for chaperone activity and filament interactions. Interactive sequences in human α B crystallin that mediate interactions with chaperone target proteins (blue), desmin (pink), GFAP (orange), and actin (green) were mapped to the surface of the 3-D homology model of human α B crystallin described previously (Ghosh & Clark, 2005; Ghosh, Estrada, & Clark, 2005). While the interactions with filaments occur in similar domains on α B crystallin, there appear to be slight differences in the surface areas used for these interactions. The interactive domains for filaments correspond to the interactive domains for unfolded chaperone target proteins.

Table 1
List of proteins and antibodies used in the protein pin array assays.

Proteins	Catalogue No.	Supplier	Molecular weight	Amount used/well
Human desmin	RDI-PRO62016	Research Diagnostics, NJ	55kDa	2.5µg
Human glial fibrillary acidic protein	GFAPI	Advanced Immunochemical, CA	52kDa	2.0µg
Rabbit muscle actin	A2522	Sigma-Aldrich, MO	43kDa	1.8µg
Antibodies	Catalogue No.	Supplier	Type	Dilution used
Anti-human desmin	RDI-PRO10519	Research Diagnostics, NJ	Mouse	1:100
Anti-human glial fibrillary acidic protein	G9269	Sigma-Aldrich, MO	Rabbit	1:1,000
Anti-chicken skeletal actin	10601	ICN Pharmaceuticals, OH	Mouse	1:100
Anti-Mouse IgG-HRP	A9044	Sigma-Aldrich, MO	Rabbit	1:40,000
Anti-Rabbit IgG-HRP	SAB-300	Stressgen, MI	Goat	1:10,000

Secondary structure composition of desmin, GFAP, and actin at 23°C and 45°C. The ultra-violet circular dichroism spectra of desmin, GFAP, and actin were deconvoluted using Dichroweb (Lobley, Whitmore, & Wallace, 2002; Whitmore & Wallace, 2004) to determine the secondary structure composition of these proteins at 23°C and after heating at 45°C for fifteen minutes. Column 1 lists the elements of secondary structure of the proteins. Columns 3 and 4 list the secondary structure of human desmin at 23°C and 45°C respectively. Columns 5 and 6 list the secondary structure of human GFAP at 23°C and 45°C respectively. Columns 3 and 4 list the secondary structure of rabbit muscle actin at 23°C and 45°C respectively. Desmin and GFAP had more helical content at 45°C than at 23°C, while the secondary structure of actin was similar at both 23°C and 45°C.

Table 2

Proteins	Desmin		GFAP		Actin	
	23°C	45°C	23°C	45°C	23°C	45°C
% helix	16%	80%	28%	40%	23%	18%
% random coil	47%	19%	53%	44%	52%	48%
% sheet	37%	1%	19%	16%	25%	34%

List of the interactive sequences in human α B crystallin involved in chaperone activity and filament interactions. Column 1 lists the structural domain in human α B crystallin where each interactive sequence is located. Column 2 lists the chaperone sequences identified previously (Ghosh, Estrada, & Clark, 2005). Columns 3-5 list the sequences in human α B crystallin for interactions with desmin, GFAP, and actin respectively. Sequences that interacted with actin at 23°C are in **BOLD**. Sequences that interacted with partially unfolded actin at 45°C are in regular font. Common α B crystallin sequences interacted with filaments and unfolded cytosolic proteins (Ghosh, Estrada, & Clark, 2005).

Table 3

Location	Interactive sequences	Desmin sequences	GFAP sequences	Actin sequences
N-terminus	⁹ WIRRPFFPHSP ₂₀	¹⁵ PPFHSPSRLF ₂₄	⁹ WIRRPFP ₁₆	¹⁵ PPFHSPSRLFDQ ₂₆
N-terminus	⁴³ SLSPFYLRPPSFLRAP ₅₈	⁴¹ STLSPPFYLRPPSFLRAPSWFD ₆₂	⁴⁵ SPFYLRPPSFLRAPSWFD ₆₂	³³ LES DLEPTSTLS SPFYLRPPSFLR ₅₆
N-terminus	-	-	-	⁵⁷ APSWFDTC ₆₄
α crystallin core domain	⁷⁵ FSVNLDVK ₈₂	⁷⁵ FSVNLDVKHFSPEELK ₉₀	⁸⁹ LKVKVLGDV ₉₈	-
α crystallin core domain	¹¹³ FISREFHR ₁₂₀	¹¹³ FISREFHRKYRIPADV ₁₂₈	¹¹³ FISREFHRKYRIPADV ₁₂₈	¹¹¹ HGFISREF ₁₁₈
α crystallin core domain	¹³¹ LTITSSLS ₁₃₈	¹³¹ LTITSSLS ₁₃₈	-	¹²⁹ DPL TITSSLS SDGV ₁₄₅
α crystallin core domain	¹⁴¹ GVLTVNGP ₁₄₈	¹⁴⁵ VNGPRKQV ₁₅₂	¹⁴³ LTVNGPRK ₁₅₀	¹⁴⁵ VNGPRKQVSG ₁₅₄
C-terminus	¹⁵⁷ ERTIPTRE ₁₆₄	¹⁶³ REEKPAVTAAPK ₁₇₄	¹⁵⁵ PERTIPTREEKPAVTA ₁₇₂	¹⁵⁵ PERTIPTREEK ₁₆₅