Interactive Domains in the Molecular Chaperone Human αB Crystallin Modulate Microtubule Assembly and Disassembly

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INTRODUCTION

Molecular chaperones are endogenous molecules that participate in the normal folding, processing, organization, and degradation of cellular proteins including cytoskeletal proteins [1–3]. Human αB crystallin is the archetype of small heat shock proteins (αHSPs) which are low molecular weight (<43 kDa) chaperones that organize and stabilize the cytoskeletal networks of microfilament and microtubule forming protein tubulin [4–16]. In the absence of stress, αHSPs interact directly with tubulin and microtubule associated proteins to promote microtubule assembly and disassembly [17–24]. A recent report suggests that high concentrations of αHSPs inhibit rather than promote microtubule assembly [25]. The systematic characterization of the interactive domains is necessary to understand the functional importance of αHSPs in assembly of cytoskeletal proteins.

In this study, the importance of five human αB crystallin interactive sequences 41STSLSPFYLRPPSFLRAP68 (ST), 73DRFSVNLVDKHFSS30 (DR), 113FISREFHR120 (FI), 131LTITSSLSSDGV142 (LT), and 156ERTIPITRE164 (ER) in the assembly/disassembly of microtubules and the thermal aggregation of tubulin were evaluated using synthetic peptides and the mutant αB crystallin confirmed the effects of αB crystallin interactive sequences 131LTITSSLSSDGV142 and 156ERTIPITRE164 on microtubule assembly and disassembly. Microtubule assembly varied with the ratio of tubulin to αB crystallin resolving the apparent contradictions in the results of an αB crystallin effect on tubulin assembly [19,21,25]. Localization of the tubulin interactive sequences on the surface of human αB crystallin collectively modulate microtubule assembly through a dynamic subunit exchange mechanism that depends on the concentration and ratio of αB crystallin to tubulin. These are the first experimental results in support of the functional importance of the dynamic subunit model of small heat shock proteins.

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**MATERIALS AND METHODS**

**Materials**

Synthetic zB crystallin peptides DRFSVNLVDKHF, STSLSPFYLPPSFLRPAR, FISREFHR, LITTTSSLSDDGV, and ERTI-PTRE were procured from Advanced ChemTech (Louisville, KY) and GenScript Corporation (Piscataway, NJ).

**Construction, expression, and purification of wt and mutant zB crystallins**

The zB crystallin mutants were constructed using the Quick-Change site-directed mutagenesis kit as described previously [29–32]. The R120G mutant is a single point mutant of the 113FISREFHR120 sequence of human zB crystallin, constructed by replacing Arg-120 with a glycine residue. The zBβ mutant was constructed by replacing the z crystallin core domain ββ sequence 135LITTTSSLS138 of human zB crystallin with the homologous ββ sequence 127SALSCSLS134 of human zA crystallin. The Δ155–165 mutant was constructed by deleting residues 135ERTIPTIRE145 from the C-terminus extension of human zB crystallin. Wy zB crystallin, R120G, zAββ, and Δ155–165 were expressed and purified as described previously [30–32].

**Microtubule assembly assays**

The effect of selected zB crystallin peptides on the in vitro assembly of tubulin into microtubules was evaluated using the Microtubule Stabilization/Destabilization Assay kit (Cytoskeleton; Denver, CO) as described previously [33]. Bovine brain tubulin was dissolved to 200 μM in 80 mM PIPES, 2 mM MgCl₂, 0.5 mM EGTA, 10 μM DAPI, 1 mM GTP pH 6.9. 8.5 μl of the tubulin was mixed with 40 μl of 80 mM PIPES, 2 mM MgCl₂, 0.5 mM EGTA, 7.4 μM DAPI, 1% Glycerol, 1 mM GTP pH 6.9 and 4.3 μl of 2 mM peptide in 2.5% DMSO, 2 mM Paclitaxel (polymerization promoter) in 100% DMSO, 15 mM CaCl₂ [polymerization inhibitor] in water, or 2.5% DMSO only. Microtubule assembly was monitored by measuring the fluorescence of DAPI, a molecule whose emission fluorescence at λ = 460 is enhanced 8-fold when it is incorporated into assembled microtubules [33]. Fluorescence of samples were continuously read on a Perkin Elmer Victor² V fluorescence plate reader (Excitation λ = 355 nm, Emission λ = 460 nm) at 37°C for 45 minutes.

The effect of wt and three mutant zB crystallins, A41–58, zAββ, and Δ155–165 on the in vitro assembly of tubulin into microtubules was evaluated using the Microtubule Stabilization/Destabilization Assay kit described above (Cytoskeleton; Denver, CO). Bovine brain tubulin was dissolved to 200 μM in 80 mM PIPES, 2 mM MgCl₂, 0.5 mM EGTA, 10 μM DAPI, 1 mM GTP pH 6.9. 8.5 μl of the tubulin was mixed with 40 μl of 80 mM PIPES, 2 mM MgCl₂, 0.5 mM EGTA, 7.4 μM DAPI, 16% Glycerol, 1.1 mM GTP pH 6.9 and 4.3 μl of 2 mM peptide in 2.5% DMSO, 2 mM Paclitaxel (polymerization promoter) in 100% DMSO, 15 mM CaCl₂ [polymerization inhibitor] in water, or 2.5% DMSO only. Microtubule assembly was monitored by measuring the fluorescence of DAPI, a molecule whose emission fluorescence at λ = 460 is enhanced 8-fold when it is incorporated into assembled microtubules [33]. Fluorescence of samples were continuously read on a Perkin Elmer Victor² V fluorescence plate reader (Excitation λ = 355 nm, Emission λ = 460 nm) at 37°C for 45 minutes.

**Microtubule disassembly assays**

The effect of zB crystallin peptides and mutants on the in vitro disassembly of microtubules was evaluated using the Microtubule Stabilization/Destabilization Assay kit described above (Cytoskeleton; Denver, CO) using methods described previously [33]. Microtubules were assembled at 37°C in the absence of zB crystallin peptides, zB crystallin proteins, and small molecules as described previously. Incubation of microtubules at 25°C results in spontaneous microtubule disassembly. To measure the effect on microtubule disassembly, 34 μM pre-formed microtubules were incubated with zB crystallin peptides (170 μM), wt and mutant zB crystallins (6.8 μM and 34 μM) at 25°C for 20 minutes. The decrease in DAPI fluorescence at λ = 460 nm was measured continuously for 20 minutes by exciting the samples at λ = 355 nm using a Perkin Elmer Victor² V fluorescence plate reader.

**Tubulin aggregation assays**

The effect of zB crystallin peptides and mutants on the thermal aggregation of tubulin was evaluated using ultra-violet spectrophotometry. Bovine brain tubulin was dissolved to 200 μM in 80 mM PIPES, 2 mM MgCl₂, 0.5 mM EGTA, pH 6.9. 4.25 μl of 0.8, 0.4, or 2 mM test peptide or protein was diluted into 40 μl of 80 mM PIPES, 2 mM MgCl₂, 0.5 mM EGTA, pH 6.9. 8.5 μl of the 200 μM tubulin was added to each sample. Samples were heated at 52°C and the absorbance at λ = 355 nm was measured continuously for 60 minutes using a Perkin Elmer UV-Visible Spectrophotometer. GTP and glycerol were not present in the samples because they induce the assembly of microtubules.

**Homology modeling**

The tubulin interactive sequences 113FISREFHR120, 135LITTTSSLS138, and 156ERTIPTIRE164 were mapped to a 3D structural model of human zB crystallin computed previously [26]. The human zB crystallin homology model was computed using the wheat hSHP16.9 X-ray crystal structure as described previously [26,34,35]. The Cz root mean square deviation between the superimposed model of human zB crystallin and the crystal structure of wheat hSHP16.9 was 3.25 Å. The model for the twenty-four subunit oligomer of human zB crystallin was computed using co-ordinates of the Methanococcus jannaschii hSHP16.5 twenty-four subunit crystal structure described previously [36].

**RESULTS**

The effects of synthetic peptides corresponding to five human zB crystallin interactive sequences on microtubule assembly were investigated (Figure 1). When 34 μM tubulin alone was incubated at 37°C, a rapid increase in DAPI fluorescence was observed due to the preferential binding of DAPI to assembled microtubules and maximum fluorescence was observed in approximately 45 minutes. The ST peptide slowed the rate of microtubule assembly by increasing the lag phase preceding the start of microtubule assembly without an effect on the amount of microtubules formed in 45 minutes. The DR peptide accelerated microtubule assembly without an effect on the total amount of microtubules formed in 45 minutes. In contrast, the FI peptide slowed microtubule assembly and decreased the amount of microtubules formed in 45 minutes. The LT and ER peptides increased both the rate of microtubule assembly and the amount of microtubules formed in 45 minutes. The effect of the LT and ER peptides was similar to Paclitaxel, a known promoter of microtubule assembly, while the effect of the FI peptide was similar but weaker than the effect of CaCl₂, a known inhibitor of microtubule assembly.

Sequences in zB crystallin that altered microtubule assembly overlapped with sequences for subunit-subunit interactions chaperone activity, and filament interactions, [26,27] (Figure 2). The overlap between zB crystallin sequences that altered microtubule assembly and zB crystallin chaperone sequences identified previously [27] suggested a functional role for zB crystallin in tubulin/microtubule stabilization. Consequently, the effects of the zB crystallin interactive sequences on microtubule
Effect of αB crystallin peptides on microtubule assembly. Samples containing tubulin and αB crystallin peptides or control molecules were excited at λ = 355 nm and the fluorescence emission of DAPI bound to assembled microtubules was recorded at λ = 460 nm. The fluorescence of the sample containing tubulin alone increased rapidly to a maximum value at 45 minutes of incubation at 37°C. The ST (N-terminal) and DR (β3) peptides had no effect on total microtubule assembly, the FI (loop) peptide inhibited microtubule assembly, while the LT (β8) and ER (C-terminal) peptides promoted microtubule assembly. The positive control, Paclitaxel, accelerated microtubule assembly, while the negative control, CaCl2,

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disassembly and tubulin aggregation were investigated (Figure 3). Pre-formed microtubules (34 μM) were incubated in the absence and presence of αB crystallin peptides and controls at 23°C to induce disassembly of microtubules. In the absence of αB crystallin peptides and controls, microtubules alone disassembled rapidly and minimum fluorescence was recorded in approximately 20 minutes. The FI and ER peptides inhibited microtubule disassembly by ~24% and 36% respectively similar to the microtubule-stabilizing molecule Paclitaxel, while the remaining peptides conferred little to no protection against the disassembly of microtubules.

The ability of the αB crystallin peptides to protect against the thermal aggregation of tubulin was determined by measuring the optical density (OD340) of 34 μM tubulin at 32°C for sixty minutes in the absence or presence of peptides and control molecules (Figure 3). In the absence of αB crystallin peptides and controls, tubulin aggregated rapidly and a maximum optical density was recorded in approximately 60 minutes. The α crystallin core domain peptides FI and LT had the strongest protective effects and decreased the aggregation of tubulin by ~42–44%. In contrast, the N-terminal peptide ST, the α crystallin core domain peptide DR, and the C-terminal peptide, ER, had weak protective effects and the aggregation of tubulin incubated with these peptides decreased by only 8–27% relative to the control. Microtubule assembly/disassembly and thermal aggregation assays identified the FI, LT, and ER peptides as interactive sequences in αB crystallin that were important for the dynamic assembly of microtubules.

Microtubule assembly and disassembly, and tubulin aggregation assays were conducted with αB crystallin mutants R120G, αAβ8, and Δ155–165, which contained mutations at sites corresponding to the FI, LT, and ER peptides respectively to confirm the results obtained with the synthetic peptides (Figure 4). Wt αB crystallin increased microtubule assembly by ~41%, had no effect on the microtubule disassembly, and decreased the thermal aggregation of tubulin by 65%. With the αB crystallin mutant R120G, which contains a single point mutation in the 113FISREFHR120 sequence, microtubule assembly and disassembly were unchanged while tubulin aggregation decreased. The αB crystallin mutant αAβ8, which contains multiple mutations at residues correspond-
Figure 2. Surface locations of the interactive sequences in αB crystallin for subunit-subunit interactions, chaperone activity, and interactions with filaments and tubulin. Interactive sequences for subunit-subunit interactions, chaperone activity, and interactions with filaments and tubulin identified by in vitro assays, mutagenesis, and pin array analysis were mapped to the N-terminal, β3-β8-β9, and C-terminal interface regions of the human αB crystallin homology model. The ST sequence is in the N-terminal extension, the DR, LT, and FI sequences are in the β3 and β8 strands and the loop of the α crystallin core domain, and the ER sequence is in the C-terminal extension. Surfaces formed by the LT (β8) and ER (C-terminal extension containing the Ile-X-Ile motif) sequences mediated subunit-subunit interactions as well as interactions with unfolded substrate proteins, filaments, and tubulin.

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Figure 3. Effect of synthetic αB crystallin peptides on microtubule assembly, disassembly, and tubulin aggregation. The DAPI fluorescence of assembled microtubules, disassembled tubulin, and tubulin aggregates in the absence of αB crystallin peptides and control additives were normalized to 1.0. The FI, LT, and ER peptides had the strongest effect on microtubule assembly/disassembly and tubulin aggregation, while ST and DR peptides had little to no effect microtubule assembly/disassembly and tubulin aggregation.

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ing to the 131LTITSSLSS sequence increased microtubule assembly, completely inhibited microtubule disassembly, and decreased tubulin aggregation. The D155–165 mutant, which lacks residues 155–165 corresponding to the ER peptide, increased microtubule assembly, and decreased both microtubule disassembly and tubulin aggregation. The results confirmed the importance of the αB crystallin sequences 113FISREFHR120, 131LTITSSLSSDGV142, and 156ERTIPITRE164 in microtubule assembly, disassembly and aggregation.

To evaluate the concentration dependence of αB crystallin on the assembly and disassembly of microtubules, a fixed amount (34 μM) of tubulin was incubated with increasing concentrations of wt αB crystallin (Figure 5). At low concentrations of wt αB crystallin, no measurable effect on microtubule assembly was observed. With increasing concentrations of αB crystallin, microtubule assembly increased to a maximum and then declined at high concentrations of αB crystallin where microtubule assembly was inhibited. With respect to the ratio of αB crystallin to tubulin, the effect on assembly of microtubules was minimal when the ratio of αB crystallin to tubulin was <1:4. When the ratio of αB crystallin to tubulin was between 1:4 and 2:1, the amount of microtubules formed was 35–94% higher than tubulin alone. Microtubule assembly was optimal when the ratio of αB crystallin to tubulin was approximately 1:2. When the ratio of tubulin to αB crystallin was >2:1 the amount of microtubules formed decreased as much as 30–63% compared to tubulin alone and no microtubules were formed when the ratio of tubulin to αB crystallin was 1:10. Wt αB crystallin stabilized microtubules in a concentration dependent manner and was most effective within a narrow concentration range.

DISCUSSION

Five interactive sequences in the sHSP and molecular chaperone, human αB crystallin participate in the stabilization of tubulin/microtubules. Individual synthetic αB crystallin peptides and full-length αB crystallin mutants either promoted or inhibited microtubule assembly and disassembly suggesting a complex mechanism for the effect of wild type αB crystallin on tubulin/microtubules. Synthetic peptides corresponding to the αB crystallin sequences 131LTITSSLSSDGV142 and 156ERTIPITRE164 promoted microtubule assembly. In contrast, the synthetic peptide corresponding to the 113FISREFHR120 sequence inhibited microtubule assembly. The remaining αB crystallin sequences 41STSLSPFYLRPPSF58 and 73DRFSLDVKHF53 had little or no effect on microtubule assembly. The results were consistent with previous reports in which full-length wt αB crystallin interacted with tubulin and modulated the assembly of tubulin into microtubules [17,21]. In thermal aggregation assays, the interactive sequences 113FISREFHR120 and 131LTITSSLSSDGV142 protected disassembled tubulin from unfolding and aggregation which was consistent with
previous reports that full-length wt αB crystallin protected tubulin from unfolding and aggregation under stress [18–20,22]. The ratio of αB crystallin to tubulin for each concentration of αB crystallin is listed at the top of the plot. For αB crystallin to tubulin ratios <1:4, microtubule assembly was unchanged at 1.0. For ratios between 1:4 and 2:1, microtubule assembly was >1.0 with maximum assembly observed at a tubulin to αB crystallin ratio of approximately 1:2.

Figure 5. Effect of αB crystallin concentration on microtubule assembly. Microtubule assembly (Y-axis) was sensitive to the concentration of wt αB crystallin (X-axis). Microtubule assembly in the absence of αB crystallin was normalized to 1.0. The ratio of αB crystallin to tubulin for each concentration of αB crystallin is listed at the top of the plot. For αB crystallin to tubulin ratios <1:4, microtubule assembly was unchanged at 1.0. For ratios >4:1, microtubule assembly was <1.0. For a αB crystallin to tubulin ratio of 10:1, microtubule assembly was undetectable.

The observation that the same αB crystallin domains interact with unfolding substrate proteins during chaperone activity and interact with tubulin during microtubule assembly is consistent with the dynamic subunit model for sHSP function. The structural importance of the LT and ER sequences in the normal dynamic assembly and disassembly of αB crystallin complexes and the functional role of the LT and ER sequences in promoting microtubule assembly further supports the dynamic subunit exchange model for sHSP function [26,34,36–41] (Figures 2 and 6). At high αB crystallin concentrations (>100 μM) and large αB crystallin:tubulin ratios (>4:1), where it is expected that αB crystallin was predominantly assembled into complexes, the LT and ER sequences in apposed αB crystallin subunits interacted with each other and were unable to promote microtubules assembly (Figure 6). In contrast, the FI sequence, which inhibited microtubule assembly, remained accessible on the surface of the complex for interactions with tubulin (Figure 6). At low αB crystallin concentrations (<8 μM) and small αB crystallin:tubulin

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ratios (1:4), the amount of \( \alpha B \) crystallin present was insufficient to modulate microtubule assembly and there was little or no effect on normal microtubule assembly. At intermediate \( \alpha B \) crystallin concentrations (8–100 µM) and \( \alpha B \) crystallin:tubulin ratios between 1:4 and 2:1, the LT and ER sequences were exposed on the surface of disassembled \( \alpha B \) crystallin subunits to stabilize microtubules and promote the assembly of additional microtubules. The overlap between interactive sites for assembly, chaperone activity, and filament interactions and their 3D organization on the surface of \( \alpha B \) crystallin subunits supports the dynamic subunit model for the physiological function of \( \alpha B \) crystallin, which involves the dynamic association, dissociation, and re-association of \( \alpha B \) crystallin with itself and target substrate proteins including tubulin. In vivo, the effect of \( \alpha B \) crystallin on microtubule assembly is determined by the dynamics of the equilibrium between free \( \alpha B \) crystallin subunits and \( \alpha B \) crystallin subunits self associated in oligomers or assembled in complexes with other protein substrates. If this interpretation is correct, measurement of the relative affinities between \( \alpha B \) crystallin subunits and selected substrates under normal and stress conditions will confirm the hypothesis that dynamic subunit assembly is responsible for the observed relationship between microtubule assembly and \( \alpha B \) crystallin concentration. Quantitative studies are being conducted using surface plasmon resonance (SPR) to test this hypothesis.

The results are consistent with the importance of sHSPs in the amyloid cascade pathway: formation of amyloid oligomers/fibrils→hyperphosphorylation of tau→disruption of tau-tubulin interactions→formation of neurofibrillary tangles (NFTs)→neurodegeneration [42–44]. Although various studies support the amyloid cascade hypothesis, the mechanism of interaction between amyloid plaques and NFTs remains uncharacterized. Although the constitutive expression of sHSPs in the normal brain is low, sHSPs including \( \alpha B \) crystallin are major constituents of amyloid plaques in Alzheimer’s disease patients [45–47]. A recent study reported that there is a marked increase in the expression of \( \alpha B \) crystallin and sHSP25 in transgenic mouse models of familial amyotrophic lateral sclerosis, Parkinson’s disease, dentato-rubral pallido-luysian atrophy and Huntington’s disease [48]. The resulting high concentration of \( \alpha B \) crystallin in response to the toxic stress of amyloid-β can destabilize microtubules. This hypothesis is consistent with the association of \( \alpha B \) crystallin with extracellular neurofibrillary tangles seen in Alzheimer’s disease patients [49] but not intracellular NFTs [45]. Microtubule stabilizers may have therapeutic value in neurodegenerative diseases such as Alzheimer’s disease where hyper-phosphorylation of the microtubule associated protein tau results in the disintegration of microtubules and the formation of NFTs [50,51].

Modulation of microtubule assembly is of great interest in the development of new cancer treatments [50,52–56]. The identifi-
cation of microtubule stabilizing peptides may have therapeutic significance in the development of novel bioactive peptides as anti-cancer agents [57,50]. Peptides that prevent microtubule disassembly can interrupt mitosis, prevent cell division, and trigger apoptosis. The effectiveness of two of the most important anti-cancer drugs, Paclitaxel and Docetaxel, which bind to microtubule and halt microtubule interaction, respectively. This mechanism of action involves stabilization of microtubules to disrupt cell division is limited by undesirable side effects including drug resistance. The zB crystallin peptides LITTTSSLSDDGV and ERTPTPRT that alter tubulin-microtubule dynamics can be developed into safe new therapeutics for cancer, Alzheimer’s disease, and tauopathies.

In summary, interactive sequences on the surface of zB crystallin that selectively recognize and stabilize tubulin can have dual effects on microtubule assembly that depend upon the zB crystallin-tubulin ratio. Favorable ratios stabilize tubulin and promote microtubule assembly and unfavorable ratios inhibit microtubule assembly. To our knowledge, this is the first experimental evidence for the functional importance of the dynamic subunit mechanism of sHSP assembly.

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Author Contributions

Conceived and designed the experiments: JG. Performed the experiments: JG SH. Analyzed the data: JG JG SH. Contributed reagents/materials/analysis tools: JG. Wrote the paper: JG JG SH.

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