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Formation of non-toxic Aβ fibrils by small heat shock protein under heat-stress conditions

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ABSTRACT

Small heat shock protein (sHsp) is a molecular chaperone with a conserved alpha-crystallin domain that can prevent protein aggregation. It has been shown that sHsps exist as oligomers (12–40 mer) and their dissociation into small dimers or oligomers is functionally important. Since several sHsps are upregulated and co-localized with amyloid-β (Aβ) in senile plaques of patients with Alzheimer's disease (AD), sHsps are thought to be involved in AD. Previous studies have also shown that sHsp can prevent Aβ aggregation in vitro. However, it remains unclear how the quaternary structure of sHsp influences Aβ aggregation. In this study, we report for the first time the effect of the quaternary structure of sHsp on Aβ aggregation using sHsp from the fission yeast *Schizosaccharomyces pombe* (SpHsp16.0) showing a clear temperature-dependent structural transition between an oligomer (30 °C) and dimer (50 °C) state. Aβ aggregation was inhibited by the oligomeric form of SpHsp16.0. In contrast, amyloid fibrils were formed in the presence of dimeric SpHsp16.0. Interestingly, these amyloid fibrils consisted of both Aβ and SpHsp16.0 and showed a low ThT intensity and low cytotoxicity due to their low binding affinity to the cell surface. These results suggest the formation of novel fibrillar Aβ amyloid with different characteristics from that of the authentic Aβ amyloid fibrils formed in the absence of sHsp. Our results also suggest the potential protective role of sHsp in AD under stress conditions.

1. Introduction

Protein aggregation, and particularly amyloid formation, has become of considerable interest since growing evidence shows that these processes are likely to be the key issues in the etiology of more than 20 disease states including Alzheimer's disease (AD) [1–4]. AD is pathologically characterized by senile plaques (SPs), neurofibrillary tangles (NFTs), and cerebrovascular amyloid angiopathy (CAA) [5]. Both SPs and CAA are formed by extracellular deposition of aggregated Amyloid-β peptides (Aβ), which includes soluble oligomers, protofibrils, and mature amyloid fibrils. Although it has been shown that soluble oligomeric species are more toxic than amyloid fibrils [6,7], recent reports showing direct fibril toxicity, or cytotoxicity related to fibril growth on lipid membranes, suggest that amyloid fibrils can also be a toxic species [8–11].

Molecular chaperones are proteins that recognize and bind to the exposed hydrophobic surfaces of non-native proteins, subsequently preventing protein aggregation and assisting with their correct folding to a native conformation [12]. Molecular chaperones are also involved in many important aspects of protein homeostasis, degradation and subcellular trafficking [13]. Consistent with this activity, it has been shown that molecular chaperones including Hsp70 and Hsp90 prevent Aβ aggregation [14].

Small heat shock protein (sHsp) is one of the ubiquitous chaperones that can bind denatured proteins and prevent irreversible protein aggregation, and thereby plays a critical role in organismal defense during physiological stress [15,16]. The sHsp family is characterized by the presence of an α-crystallin domain, a stretch of 80–100 amino acids in the C-terminal half of the proteins. The sHsps exist as oligomers in a functionally important dynamic equilibrium with dimers or other small complexes [15–20]. The sHsp family comprises several sHsps, including αB-crystallin and Hsp27. In AD, αB-crystallin and Hsp27 are upregulated and expressed by astrocytes surrounding SPs and NFTs [14]. Previous reports have shown that these sHsps are able to prevent mature Aβ fibril formation [21–25]. However, the quaternary structural state of these sHsp was not described in these studies, and the effect

Abbreviations: AD, Alzheimer's disease; SPs, senile plaques; NFTs, neurofibrillary tangles; CAA, cerebrovascular amyloid angiopathy; Aβ, amyloid-β peptides; sHsp, small heat shock protein; SpHsp16.0, sHsp from fission yeast *Schizosaccharomyces pombe*.

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of a disassembled dimeric state of these sHsp on Aβ aggregations remains unclear.

In order to study the relation between the quaternary structure of sHsp and its effect on Aβ aggregation, an sHsp from the fission yeast *S. pombe*, SpHsp16.0, was used in this study. Previous results showed that SpHsp16.0 exists as a hexadecameric globular oligomer near the physiological growth temperatures (25–38 °C), whereas the oligomer dissociated into dimers at 50 °C [19,20]. The dissociation was completely reversible, and the original oligomer reformed immediately after the temperature dropped. The sequence similarity of SpHsp16.0 with human Hsp27 is approximately 60%. Therefore, SpHsp16.0 may represent a suitable sHsp model to study the effect of its quaternary structure on Aβ aggregations.

2. Materials and methods

2.1. Materials

Synthetic wild-type $A\beta_{1-42}$ (Aβ), ThT and RPMI 1640 medium were purchased from Sigma (MO, USA). SpHsp16.0 was prepared as described previously [20]. Mouse monoclonal antibodies to Aβ (6E10 and 4G8) were purchased from Abcam (Cambridge, UK) and Chemicon (CA, USA), respectively. Rabbit polyclonal antibody to SpHsp16.0 was prepared by Technical Keystone Craft (Gunma, Japan). HRP conjugated with anti-rabbit immunoglobulin G (IgG) and HRP conjugated anti-mouse IgG were supplied by R&D systems (MN, USA). Alexa488-conjugated anti-rabbit IgG and Alexa555-conjugated anti-mouse IgG were purchased from Molecular Probes (OR, USA). The enhanced chemiluminescence (ECL) and western blotting detection systems were obtained from GE Healthcare (Little Chalfont, UK). The cell proliferation kit (MTT) was purchased from Roche (IN, USA). The VECTASHIELD HardSet Mounting Medium with DAPI was supplied by VECTOR Laboratories (CA, USA).

2.2. Preparation of Aβ aggregates

Aβ peptide was dissolved in a 0.02% ammonia solution at 500 μM. To obtain a seed-free Aβ solution, the prepared solution was centrifuged at 100,000 rpm for 3 h to remove pre-existing seeds [26]. The supernatant was collected and stored at −80 °C. A 30 μM Aβ peptide sample was incubated with or without 30 μM SpHsp16.0 in PBS buffer at 30 or 50 °C for 24 h. The conversion ratio from Aβ monomer into Aβ amyloid fibrils was calculated by measuring the amount of unconverted Aβ remaining in the supernatants with an ELISA assay after centrifugation (15,000 rpm, 20 min) of part of the incubated Aβ samples.

2.3. Transmission electron microscopy (TEM)

The sample was placed on a carbon-coated copper grid and allowed to absorb. Excess sample was removed from the grid using filter paper, and the grid was air dried before negative staining with uranyl acetate. Excess stain was then removed from the grid by air drying. Samples were observed at a 100 kV excitation voltage using a JEM-1011 TEM (JEOL, Tokyo, Japan).

2.4. ThT binding assay

Aβ fibrillation was assessed by the ThT assay as already described [6,27]. Aβ samples (0.25 μM) were added to 50 mM glycine-NaOH (pH 8.5) buffer containing 5 μM ThT. Each sample was excited at 445 nm, and the emission was recorded at 482 nm on a spectrofluorometer (FP-6500, Jasco, Tokyo, Japan).

2.5. Immunoprecipitation

The Aβ samples (100 μl) incubated with SpHsp16.0 at 30 or 50 °C were diluted to a volume of 1 ml and incubated with 10 μl anti-Aβ (4G8) or 10 μl anti-SpHsp16.0 overnight at 4 °C with rotating. The samples were then incubated with 100 μl of Protein G-Sepharose beads (20% slurry in PBS, Amersham, Amersham, UK) for 7 h at room temperature. All anti-Aβ or SpHsp16.0-bound material was pulled down by centrifugation of samples, and separating the beads from the supernatant. The immunoprecipitates were washed 3 times with PBS, and thereafter incubated with SDS sample buffer (40 μl) at room temperature for 20 min. Eluted proteins (3 μl) were blotted on nitrocellulose membrane, and analyzed with western blotting using anti-SpHsp16.0 for samples immunoprecipitated by anti-Aβ(4G8), or anti-Aβ (6E10) for samples immunoprecipitated by anti-SpHsp16.0, using the ECL Plus blotting detection system (GE Healthcare). The incubated Aβ-alone or SpHsp16.0-alone samples were used as controls. For a control experiment, a similar immunoprecipitation was performed on the insoluble fraction after centrifugation (15,000 rpm, 20 min) of incubated Aβ samples.

2.6. Toxicity assay

Cell viability was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay according to the manufacturer's instructions (Roche) as already described [8,28]. Rat PC12 cells (ATCC, VA, USA) (5×10^4/well) were plated in 96-well plates coated with poly-D-lysine, and covered with 100 μl culture medium. After plating, 20 μl medium was removed from each well, and replaced with the same volume of Aβ samples (0.5 μM) incubated with or without SpHsp16.0 at 30 or 50 °C for 24 h. The MTT solution was then added to each well and incubated for a further 4 h. The adsorption values at 550 nm were determined with an Appliskan microplate reader (Thermo Scientific, Kanagawa, Japan).

2.7. Immunostaining

PC12 cells (2.5×10^5 cells/well) were incubated in a slide chamber overnight in humidified 5% CO_2 incubators. Aβ samples (0.5 μM) incubated with or without spHsp16.0 at 30 or 50 °C for 24 h were then added to cells, and further incubated at 37 °C for 24 h. After removal of the medium, cells were fixed in formaldehyde, and blocked with 10% donkey serum in TBST for 1 h. Cells were incubated with mouse anti-Aβ (6E10, 1:1000) or rabbit anti-spHsp16.0 (1:4000), and then with Alexa 488 or 555 conjugated anti-mouse or anti-rabbit IgG (1:250) at room temperature for 1 h and washed three times with TBST. Slides were examined under a fluorescent microscope (Axio Observer Z1, Carl Zeiss).

3. Results and discussion

3.1. Fibrillation of Aβ peptide in the presence of sHsp

It has been shown that SpHsp16.0 dissociates into a dimer under heat shock conditions (50 °C), while it exists as a hexadecamer at a normal temperature (30 °C) [20]. In an effort to investigate the effects of sHsp structure on fibrillation of $A\beta_{1-42}$ peptide, the major factor responsible for AD, Aβ fibrillation in the presence of an equimolar (monomer conc.) amount of SpHsp16.0 at 30 or 50 °C for 24 h was examined using TEM (Fig. 1A). In the absence of SpHsp16.0, formation of amyloid fibrils was observed both at 30 and 50 °C. The conversion ratio from Aβ monomer into Aβ fibrils was calculated as 100% when measuring the amount of uncon-

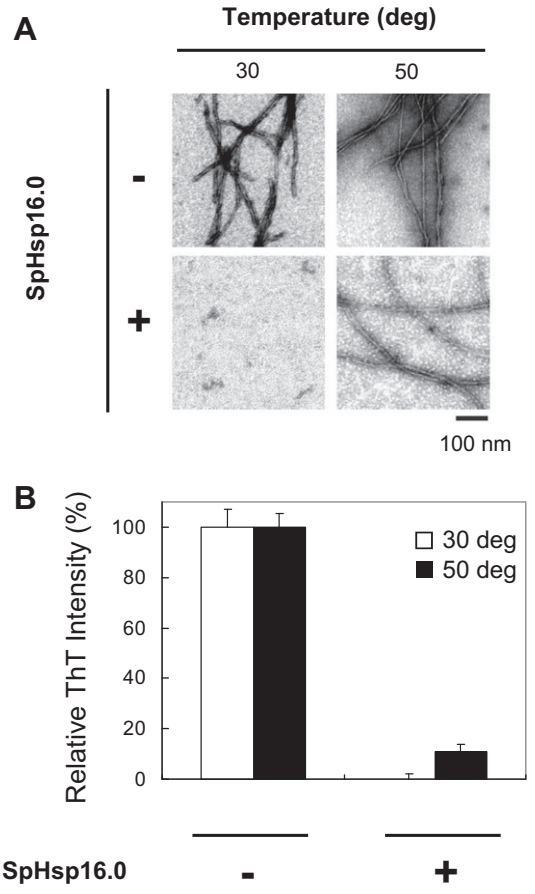

Fig. 1. Fibrillation of Aβ peptide in the presence of SpHsp 16.0 at 30 °C (oligomer) and 50 °C (dimer). (A) TEM analysis. Scale bar = 100 nm. (B) ThT assay. Aβ samples incubated in the absence or presence of SpHsp 16.0 at 30 °C (white, oligomer) and 50 °C (black, dimer) were added to ThT solution, and ThT fluorescence at 482 nm was measured. The intensities for Aβ samples incubated without SpHsp16.0 were normalized as 100%.

verted Aβ monomer remaining in the supernatants after centrifugation of part of the incubated Aβ samples.

By contrast, when Aβ was incubated with an equimolar amount of SpHsp16.0 at 30 °C, no mature amyloid fibrils were formed. The conversion ratio of Aβ monomer to the insoluble fraction was determined as 0%, indicating that all Aβ molecules remained in a soluble state. Intriguingly, however, amyloid fibrils were observed in samples incubated with SpHsp16.0 at 50 °C. The conversion ratio was 75%. These results were not expected because our previous study showed that SpHsp16.0 exhibited chaperone activity in the dissociated state at the elevated temperature [20]. It is plausible that oligomeric SpHsp16.0 is able to prevent aggregation of small proteins such as Aβ (4.5 kDa), since oligomeric SpHsp16.0 could also prevent DTT-induced aggregation of the insulin peptide (5.8 kDa) at 30 °C (Supplemental Figure.).

Aβ fibrillation was also examined using ThT fluorescence (Fig. 1B). When the Aβ peptide was incubated with SpHsp16.0 at 30 °C, only a trace level of ThT fluorescence was observed, which is consistent with the TEM observation (Fig. 1A). Interestingly, the ThT fluorescence for Aβ fibrils formed in the presence of SpHsp16.0 at 50 °C decreased to approximately 10% of that without SpHsp16.0 (Fig. 1B). As the conversion ratio of Aβ monomer into the insoluble fraction was high enough (75%), this result indicates that binding of ThT to the amyloid fibril incubated with the SpHsp16.0 dimer is weak. Therefore, this finding suggests that the inner structure is different from the morphologically similar authentic amyloid fibrils.

3.2. Interaction between SpHsp16.0 and Aβ

The results obtained with TEM and the ThT assay suggest an interaction between Aβ molecules and SpHsp16.0 in both oligomeric (30 °C) and dimeric (50 °C) structures. Their interaction was further confirmed with co-immunoprecipitation (Fig. 2). Samples were immunoprecipitated with anti-Aβ (4G8) bound to Sepharose beads, followed by detection with anti-SpHsp16.0 (lane 1). This result indicates that Aβ molecules form a complex with SpHsp16.0 of both dimeric (50 °C) and oligomeric (30 °C) structures. The negative control experiments without one of the antibodies confirm the absence of cross-reactivity. Similar results were obtained for samples immunoprecipitated with anti-SpHsp16.0 and detected with anti-Aβ (6E10) (lane 2).

Immunoprecipitation was also performed on the insoluble fraction after centrifugation of incubated samples (lane 3 and 4). The observed interaction between SpHsp16.0 and Aβ molecules in samples incubated at 50 °C confirmed the formation of Aβ amyloid complex with SpHsp16.0. By contrast, no SpHsp16.0 or Aβ molecules were observed in the insoluble fraction of the sample incubated at 30 °C. It is plausible that Aβ molecules were protected from amyloid formation and remained soluble due to the interaction with oligomeric SpHsp16.0.

3.3. Cytotoxicity of Aβ samples incubated with SpHsp16.0

Aβ amyloid has been shown to be cytotoxic and is considered one of the causative agents of AD. We therefore examined the cytotoxicity of Aβ samples incubated with SpHsp16.0 using the MTT assay (Fig. 3A). Cell death was observed upon addition of 0.5 μM Aβ amyloid fibrils formed in the absence of SpHsp16.0 at 30 °C or 50 °C. By contrast, the same volume of aliquots of Aβ samples incubated with SpHsp16.0 at 30 °C showed only a little cell toxicity, which is consistent with the results obtained by TEM (Fig. 1A) and the ThT assay (Fig. 1B) showing suppression of the formation of insoluble Aβ aggregates by the oligomeric SpHsp16.0. This finding also suggests that no toxic Aβ oligomer was formed. Similar results were obtained for insoluble fractions of Aβ samples after centrifugation (15,000 rpm, 10 min) (data not shown).

Interestingly, Aβ aggregates formed in the presence of SpHsp16.0 at 50 °C also showed only a little cell toxicity. Combined with the TEM result (Fig. 1A), this finding indicates that fibrillar Aβ amyloids formed in the presence of dimeric SpHsp16.0 have significantly lower cytotoxicity than the authentic Aβ amyloid irrespec-

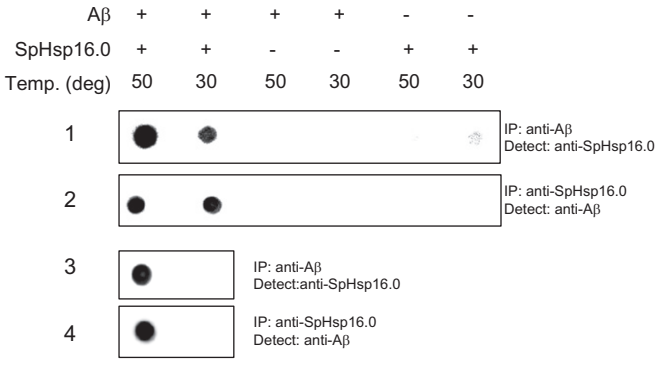

Fig. 2. Immunoprecipitation of Aβ samples incubated in the presence of SpHsp16.0 at 30 °C (oligomer) and 50 °C (dimer). Lane 1, Immunoprecipitated with anti-Aβ antibody and detected with anti-SpHsp 16.0 antibody; Lane 2, Immunoprecipitated with anti-SpHsp16.0 and detected with anti-Aβ; Lane 3, Immunoprecipitation of the insoluble fraction of a co-incubated sample with anti-Aβ and detected with anti-SpHsp16.0; Lane 4, Immunoprecipitation of the insoluble fraction of a co-incubated sample with anti-SpHsp 16.0 and detected with anti-Aβ.

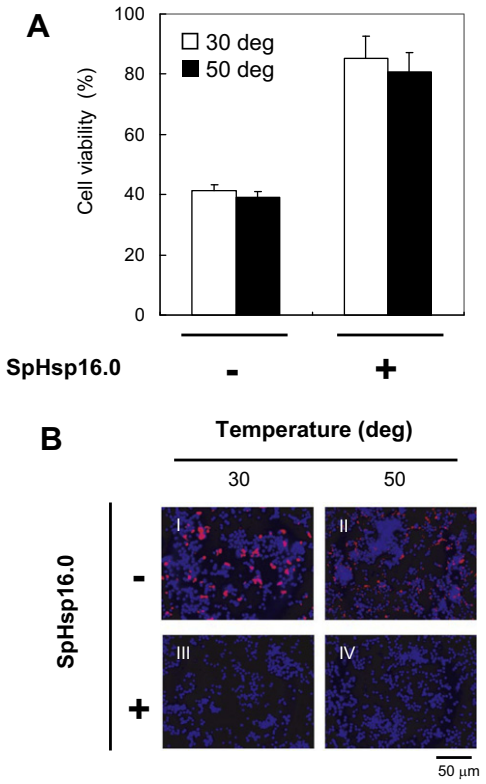

Fig. 3. Cytotoxicity of Aβ samples incubated in the presence of SpHsp16.0 at 30 °C (oligomer) and 50 °C (dimer). (A) Cell viability was determined with the MTT assay. PC12 cells were incubated with 0.5 μM Aβ samples incubated in the absence or presence of SpHsp 16.0 at 30 °C (white, oligomer) and 50 °C (black, dimer). (B) Binding of Aβ samples on PC12 cells. Aβ samples (0.5 μM) incubated with or without spHsp16.0 at 30 or 50 °C for 24 h were added to PC12 cells, and further incubated at 37 °C for 24 h. After removal of the medium, cells were incubated with mouse anti-Aβ (1:1000), and then with Alexa 555 conjugated anti-mouse IgG (1:250). Slides were examined under a fluorescent microscope (Red, Aβ; Blue, DAPI). Scale bar = 50 μm.

tive of the morphological similarity. The ThT assay also shows the difference between Aβ amyloid fibril incubated with dimeric SpHsp16.0 and authentic Aβ amyloid fibrils (Fig. 1B).

The interaction between the cell surface and Aβ aggregates formed in the presence of SpHsp16.0 was examined with immunostaining (Fig. 3B). PC12 cells were incubated with 0.5 μM Aβ fibrils formed in the absence of SpHsp16.0 at 30 °C or 50 °C, and in the presence of SpHsp16.0 at 50 °C for 24 h at 37 °C. After washing out unbound Aβ samples and fixing cells on the dishes, Aβ bound on the cell surface was recognized with anti-Aβ (6E10) and visualized with Alexa555 labeled secondary antibody. As shown in Fig. 3B, Aβ aggregates formed in the absence of SpHsp16.0 were observed bound to the cell (panels I and II), a finding that is consistent with the high cell toxicity. Since cells were not subjected to any detergent treatment, this result indicates that Aβ aggregates exist on the cell surface and not inside the cell. By contrast, Aβ aggregates formed in the presence of dimeric SpHsp16.0 (50 °C) were not observed on the cell surface (panel IV). It is therefore plausible that the novel Aβ aggregates were not cytotoxic due to their low binding affinity to the cell surface. This is consistent with our previous results showing correlation between amyloid toxicity and cell binding [8]. No Aβ molecule was observed on the cell surface for the Aβ sample incubated with oligomeric SpHsp16.0 (30 °C, panel III), a finding that is also consistent with the previous results in this study indicating suppression of Aβ aggregation by oligomeric sHsp [21–25].

3.4. Characteristics of the Aβ amyloid-SpHsp16.0 complex

These data suggest that Aβ amyloids formed in the presence of dimeric SpHsp16.0 differ from authentic Aβ amyloids. In order to elucidate its mechanism of formation, dimeric SpHsp16.0 was added to pre-formed Aβ amyloids. If their characteristics are similar, it is plausible that dimeric SpHsp16.0 binds to the surface of Aβ amyloids.

Aβ peptide (30 μM) was incubated at 50 °C for 24 h as described above. After the removal of soluble fractions of Aβ amyloid aggregates by centrifugation, 30 μM dimeric SpHsp16.0, which was formed by incubation at 50 °C for 10 min, was added and further incubated at 50 °C for 10 min. Complex formation between the pre-formed Aβ amyloids and dimeric SpHsp16.0 was confirmed with co-immunoprecipitation (Fig. 4A). Samples were immunoprecipitated with anti-Aβ (4G8) bound to Sepharose beads, and SpHsp16.0 was detected with anti-SpHsp16.0. As shown in Fig. 4A (lane 1), SpHsp16.0 bound to the pre-formed Aβ amyloids was detected, which indicates that the dimeric SpHsp16.0 binds to the pre-formed Aβ amyloids. Formation of a complex between these components was confirmed with co-immunoprecipitation using different antibodies (immunoprecipitated with anti-SpHsp16.0 and detected with anti-Aβ (6E10)) (Fig. 4A, lane 2).

The pre-formed Aβ amyloid-dimeric SpHsp16.0 complex was also examined with ThT fluorescence (Fig. 4B). Addition of dimeric SpHsp16.0 did not affect the ThT intensity of the pre-formed Aβ amyloids, which is different from the Aβ amyloids formed in the presence of dimeric SpHsp16.0 (Fig. 1B). This result therefore indirectly suggests that dimeric SpHsp16.0 is involved in the formation of novel hybrid amyloid composed of SpHsp16.0 and Aβ when they are co-incubated.

Cytotoxicity of the pre-formed Aβ amyloid-dimeric SpHsp16.0 complex was examined with the MTT assay (Fig. 4C). Results revealed that the cytotoxicity of this complex was similar to that of Aβ amyloids, indicating that the bound SpHsp16.0 does not affect the cytotoxicity of Aβ amyloids.

Interaction between the PC12 cell surface and the pre-formed Aβ amyloid-dimeric SpHsp16.0 complex was examined with immunostaining (Fig. 4D). Complex formation between dimeric SpHsp16.0 and the pre-formed Aβ amyloid was also confirmed (panel IV). Consistent with the cytotoxicity result, SpHsp16.0-preformed Aβ complex bound on the cell surface was detected. This result also supports the difference between dimeric SpHsp16.0-pre-formed Aβ amyloid complex and the novel SpHsp16.0-Aβ amyloid complex formed in the presence of dimeric SpHsp16.0.

The overall results support our view that the Aβ amyloids formed in the presence of dimeric SpHsp16.0 did not represent a complex between SpHsp16.0 and Aβ amyloid, but novel SpHsp16.0-Aβ hybrid amyloids. Previous reports by Meehan et al. showed that human αB-crystallin proteins, one of the sHsps that has 59% similarity with SpHsp16.0, form amyloid fibrils under mildly denaturing condition (incubation at 60 °C for 24 h in the presence of 1 M guanidine hydrochloride) [29], suggesting a potential amyloid formation property for sHsps. Although no amyloid was formed by SpHsp16.0 in our conditions, it is plausible that SpHsp16.0 was utilized during Aβ amyloid formation. Further studies will be necessary to validate this hypothesis.

In conclusion, we report for the first time the effect of the quaternary structure of sHsp on Aβ aggregation using yeast sHsp (SpHsp16.0) showing a clear temperature-dependent structural transition between an oligomer and dimer state. Aβ aggregation was inhibited by the oligomeric form of SpHsp16.0. In contrast, amyloid fibrillar aggregation was formed in the presence of dimeric SpHsp16.0. Interestingly, this fibrillar amyloid aggregation, consisting of both Aβ and SpHsp16.0, showed low ThT intensity and low cytotoxicity due to a low binding affinity to the cell sur-

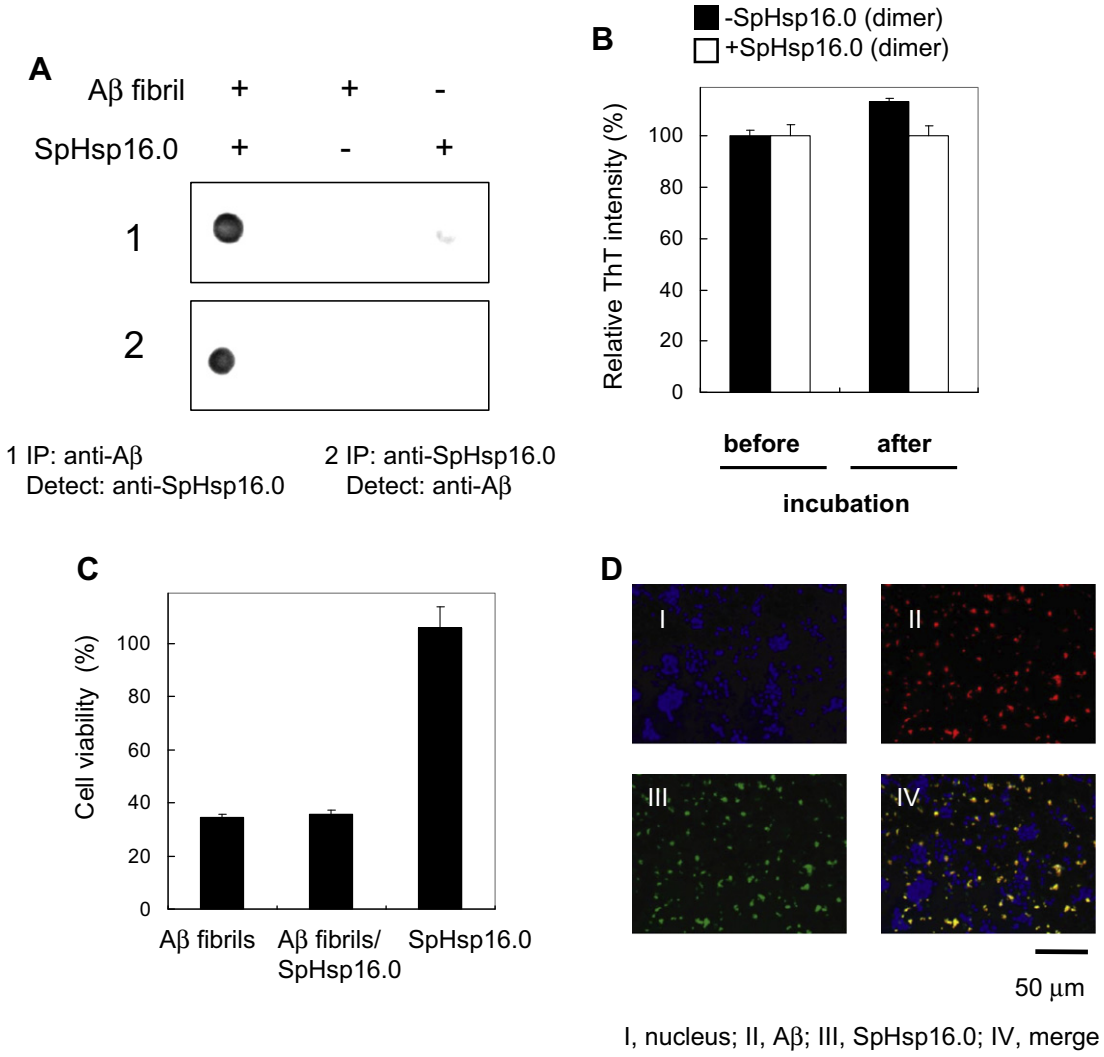

Fig. 4. Characteristics of preformed-Aβ amyloid and SpHsp16.0 complex. Aβ amyloid fibrils were first formed and then dimeric SpHsp16.0 incubated at 50 °C was added to form a complex. (A) Immunoprecipitation. Samples were immunoprecipitated with anti-Aβ (4G8), and detected with anti-SpHsp16.0 (lane 1), or vice versa (lane 2). Aβ amyloid fibril-only and SpHsp16.0-only samples were used as control samples. (B) ThT assay. ThT intensities of Aβ amyloids before and after incubation with dimeric SpHsp16.0 are shown. PBS was used as a control (black). (C) Cytotoxicity of preformed-Aβ amyloid and SpHsp16.0 complex. PC12 cells were incubated with 0.5 μM Aβ fibrils incubated with or without dimeric SpHsp16.0. Cytotoxicity of SpHsp16.0 is also shown as a control. (D) Binding of preformed-Aβ amyloid and SpHsp16.0 complex on PC12 cells. Aβ sample (0.5 μM) incubated with dimeric SpHsp16.0 was added to PC12 cells, and further incubated at 37 °C for 24 h. After removal of the medium, cells were incubated with mouse anti-Aβ (1:1000) and goat anti-SpHsp16.0 (1:4000), and then with Alexa 555 or 488 conjugated anti-mouse or anti-rabbit IgG (1:250). Slides were examined under a fluorescent microscope (red, Aβ; green, SpHsp16.0; blue, DAPI). Scale bar = 50 μm.

face. We also showed with controlled experiment using preformed Aβ amyloid that Aβ amyloids formed in the presence of dimeric SpHsp16.0 did not represent a complex between SpHsp16.0 and Aβ amyloid, but might be novel SpHsp16.0-Aβ hybrid amyloids. Our results also suggest the potential protective role of sHsp in AD. Further studies will be necessary to validate these hypotheses.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.12.059.

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