Human prefoldin can inhibit BETA-AMYLOID fibrillation and contribute to formation of non-toxic BETA-AMYLOID aggregates

ARTICLE *in* ALZHEIMER'S AND DEMENTIA · JULY 2012

Impact Factor: 12.41 · DOI: 10.1016/j.jalz.2013.08.136

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Human Prefoldin Inhibits Amyloid-β (Aβ) Fibrillation and Contributes to Formation of Nontoxic Aβ Aggregates

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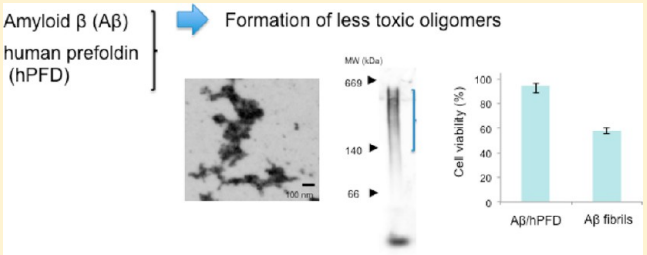
ABSTRACT: Amyloid-β (Aβ) peptides represent key players in the pathogenesis of Alzheimer's disease (AD), and mounting evidence indicates that soluble Aβ oligomers mediate the toxicity. Prefoldin (PFD) is a molecular chaperone that prevents aggregation of misfolded proteins. Here we investigated the role of PFD in Aβ aggregation. First, we demonstrated that PFD is expressed in mouse brain by Western blotting and immunohistochemistry and found that PFD is upregulated in AD model APP23 transgenic mice. Then we investigated the effect of recombinant human PFD (hPFD) on Aβ(1−42) aggregation *in vitro* and found that hPFD inhibited Aβ fibrillation and induced formation of soluble Aβ oligomers. Interestingly, cell viability measurements using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay showed that Aβ oligomers formed by hPFD were 30−40% less toxic to cultured rat pheochromocytoma (PC12) cells or primary cortical neurons from embryonic C57BL/6CrSlc mice than previously reported Aβ oligomers (formed by archaeal PFD) and Aβ fibrils ($p < 0.001$). Thioflavin T measurements and immunoblotting indicated different structural properties for the different Aβ oligomers. Our findings show a relation between cytotoxicity of Aβ oligomers and structure and suggest a possible protective role of PFD in AD.



Alzheimer's disease (AD) is pathologically characterized by senile plaques, synapse loss, and accumulation of neurofibrillar tangles in the brain.[1] Senile plaques consist of amyloid-β (Aβ) peptides, which are 40−43 amino acids in length and released from the amyloid precursor protein (APP). Aβ is produced in cultured cells and *in vivo* during normal cell metabolism;[2,3] thus, the presence of Aβ is not a sign of disease itself. Aβ is unstable and aggregates easily once produced. Recent studies have shown a robust correlation between the soluble Aβ oligomer levels and the extent of synaptic loss or severity of cognitive impairment.[4−11] These soluble Aβ oligomers are also toxic to cultured cells.[12,13] By "soluble oligomers", we refer to any dimeric or larger oligomeric structure that is soluble in aqueous solution and remains in solution after high-speed centrifugation.

The structures and sizes of soluble Aβ oligomers vary widely,[14−20] and it remains unclear which oligomeric species represent the toxic form(s). Small, globular Aβ oligomers, termed Aβ-derived diffusible ligands (ADDLs), are reported to play a role in AD pathology.[15,16] However, other constitutions of Aβ are known to be toxic to cells and also to play a role in AD; these include large, spherical structures, termed amylospheroids,[17] and large, spherical Aβ aggregates formed in the presence of liposomes containing GM1 ganglioside.[14] O'Nullain and colleagues recently reported that dimers aggregated rapidly and formed protofibrils that could block long-term potentiation.[19] Takamura et al. found that extracellular and intraneuronal high-molecular weight (HMW) Aβ oligomers caused neuronal degeneration.[18] Hence, several conformations of soluble Aβ oligomers might contribute to neuronal death in AD.

Molecular chaperones represent a diverse group of proteins that recognize and bind unfolded proteins, act to prevent their misfolding and aggregation, and assist their transition to a native conformation.[21−24] The ability of molecular chaperones to prevent off-pathway folding events and to refold proteins that have lost their natural conformation makes them key players in studies of protein misfolding diseases and potential therapeutic agents.[25,26] Indeed, several publications have demonstrated possible roles for molecular chaperones in AD pathology.[27−29] For example, the small heat shock protein αB-crystallin could inhibit Aβ fibrillation and the associated cytotoxicity of Aβ.[27] Cortical neurons expressing Hsp27 were protected against neuronal degeneration caused by Aβ treatment in culture medium,[28] and HspB1 could sequester toxic Aβ oligomers and convert them into large nontoxic aggregates.[29]

Received: December 25, 2012
Revised: April 20, 2013
Published: April 24, 2013

In this study, we investigated the potential role of prefoldin (PFD) in modulating the oligomerization pathway and cytotoxicity of Aβ peptide. PFD is a molecular chaperone conserved in archaea and eukaryotes.[30−32] In the eukaryotic cytosol, it interacts with and stabilizes nascent polypeptides. Eukaryotic PFD is believed to bind to unfolded actins and tubulins and possibly other proteins and to transfer them to cytosolic chaperonin containing TCP-1 (CCT) for folding to the native state.[30,31,33] PFD consists of six subunits, and the subunits can be grouped into two evolutionarily related classes: α and β. Two eukaryotic α-subunits (PFD3 and PFD5) and four β-subunits (PFD1, PFD2, PFD4, and PFD6) exist. Functional PFD hexamers assemble via two β hairpins in the α class subunit and one β hairpin in the β class subunits.[32,34] Both human and archaeal PFD possess quaternary structures resembling a jellyfish with six "tentacles" consisting of coiled coils that interact with and stabilize non-native polypeptides.[34−36]

We previously showed that Aβ fibrillation was inhibited in the presence of archaeal PFD from *Pyrococcus horikoshii* (PhPFD) and that Aβ incubated in the presence of PhPFD formed HMW soluble oligomers that were toxic to cultured rat pheochromocytoma (PC12) cells.[37] In this study, we investigated the potential role of human PFD (hPFD) in modulating Aβ oligomerization and cytotoxicity. First, we confirmed the presence of PFD in mouse brain by immunostaining of brain sections from wild-type (wt) and APP23 transgenic (tg) mice (AD mouse model with 7-fold overexpression of APP and an increasing level of Aβ deposits with age[38]) for PFD. We next investigated hippocampal and cortical brain homogenate of wt and APP23 tg mice and found that PFD was upregulated in APP23 tg mice. Analysis of Aβ and hPFD *in vitro* revealed that Aβ fibrillation was inhibited in the presence of hPFD, as confirmed by Western blot analysis and transmission electron microscopy (TEM). Furthermore, cell viability tests using PC12 cells, or primary cortical neurons, revealed the significantly reduced toxicity of Aβ oligomers that had been incubated with hPFD, in contrast to PhPFD-formed Aβ oligomers.[37] We also demonstrated that nontoxic Aβ oligomers formed by hPFD and toxic Aβ oligomers formed by PhPFD exhibited different antibody recognition properties, as well as different ThT binding properties, which suggests that differences in surface structures might be important for oligomer toxicity.

MATERIALS AND METHODS

Immunohistochemical Studies of PFD Expression in Mouse Brain. Brains from APP23 tg[38] or wt mice were fixed by cardiac perfusion with PBS, incubated overnight in 4% PFA, processed, and embedded in paraffin. Paraffin-embedded 4 μm thick sections were cut and analyzed by immunostaining using goat polyclonal anti-hPFD subunit 5 (hPFD5) (Santa Cruz Biotechnology, Santa Cruz, CA) and secondary anti-goat with tyramide signal amplification (PerkinElmer Life Sciences). The cell nuclei were stained with Hoechst-33342 (Calbiochem, San Diego, CA). As control samples, secondary antibodies with tyramide signal amplifications were added directly to tissues without addition of primary antibodies beforehand (data not shown).

Homogenization of Mouse Brain and Western Blotting. Brains from APP23 tg or wt mice were homogenized with a multibead shocker, metal cone (MC-0214R) at 2500 rpm for 20 s (one cycle) and ultracentrifuged at 70000 rpm for 29 min. Five volumes of homogenization buffer [50 mM Tris-HCl, 150 mM NaCl (pH 7.4), Complete (EDTA plus), and pepstatin A] per brain was used. For Western blotting, three different individuals from either APP23 tg- or wt mice were tested. The homogenate (with protein concentrations normalized to 2 μg/μL) from each individual was mixed with an equal volume of sample buffer and subjected to 15% (w/v) sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). The proteins were transferred to a PVDF membrane (Hybond P, GE Healthcare, Little Chalfont, U.K.). After being blocked with 2% ECL Advance blocking agent (GE Healthcare) in 0.1% PBST at 4 °C overnight, the membrane was probed with anti-hPFD5 (1:50) and mouse anti-actin (1:2000) (Sigma, St. Louis, MO). Image J was used to quantify the intensities of the bands. The intensities in wt mice were normalized as 100%.

Expression and Purification of hPFD. The hPFD subunits were expressed in *Escherichia coli* BL21(DE3), purified as described previously,[39] and stored in buffers that included 20% glycerol at −80 °C. Upon being used, the subunits were assembled to a hexameric complex.[39] Briefly, the subunits were combined at equimolar amounts (4−8 nmol) and brought to a final urea concentration of 1.5 M. The mixture was dialyzed against PBS for 1.5 h, fractionated via gel filtration, and analyzed via 15% SDS−PAGE to confirm that the obtained complex included all subunits.

Preparation of Aβ Aggregates. Aβ(1−42) peptide was purchased from Peptide Institute (Osaka, Japan), and seed-free Aβ solutions were prepared as described previously.[14] Briefly, the Aβ peptide was dissolved in a 0.1% ammonia solution at 1.25 mg/mL and ultracentrifuged at 100000 rpm for 3 h to obtain a seed-free Aβ solution.[14] Then the supernatant was collected and stored as aliquots at −80 °C at a concentration of 170 μM (0.77 mg/mL). Before being used, Aβ was thawed, diluted with PBS, and incubated at 25 μM and 37 °C for 48 h, in the absence or presence of various levels of hPFD (1 nM to 5 μM). For a comparison study using archaeal PhPFD, 50 μM Aβ was incubated with 50 μM PhPFD at 50 °C (which is the temperature at which PhPFD is active) for 48 h, which is the appropriate condition for the formation of oligomers as described previously.[37] After incubation, samples were diluted to appropriate concentrations (and equivalent Aβ concentrations for the comparison of Aβ and hPFD vs Aβ and PhPFD) for the following experiments. For Aβ fibril formation, 25 μM Aβ was incubated for 48 h at 37 °C.

Cell Culturing. PC12 cells were cultured in RPMI 1640 medium (Sigma) supplemented with 10% horse serum, 5% fetal bovine serum, 0.1% penicillin, and 0.1% streptomycin on poly-D-lysine (PDL)-coated dishes. For neurite formation, PC12 cells were passaged to PDL-coated dishes to a density of 20000 cells/well and NGF (Invitrogen, Carlsbad, CA) was added to a final concentration of 100 ng/mL. The cells were cultured until they were ready to use as described previously.[14]

Primary cortical neurons were prepared from embryonic C57BL/6CrSlc mice from a developmental state of 16−18 days, according to a previously described method.[40] Briefly, the cells were isolated, washed, and cultured in Neurobasal medium with 2% B27, 0.5 mM glutamine, and penicillin/streptomycin, at a density of 50000 cells/well on poly-L-lysine (PLL)-coated 96-well plates. Primary cortical neurons were cultured for 14 days to allow neurite formation.

Cell Viability Measurements. The viability of PC12 cells and primary cortical neurons was measured using Cell

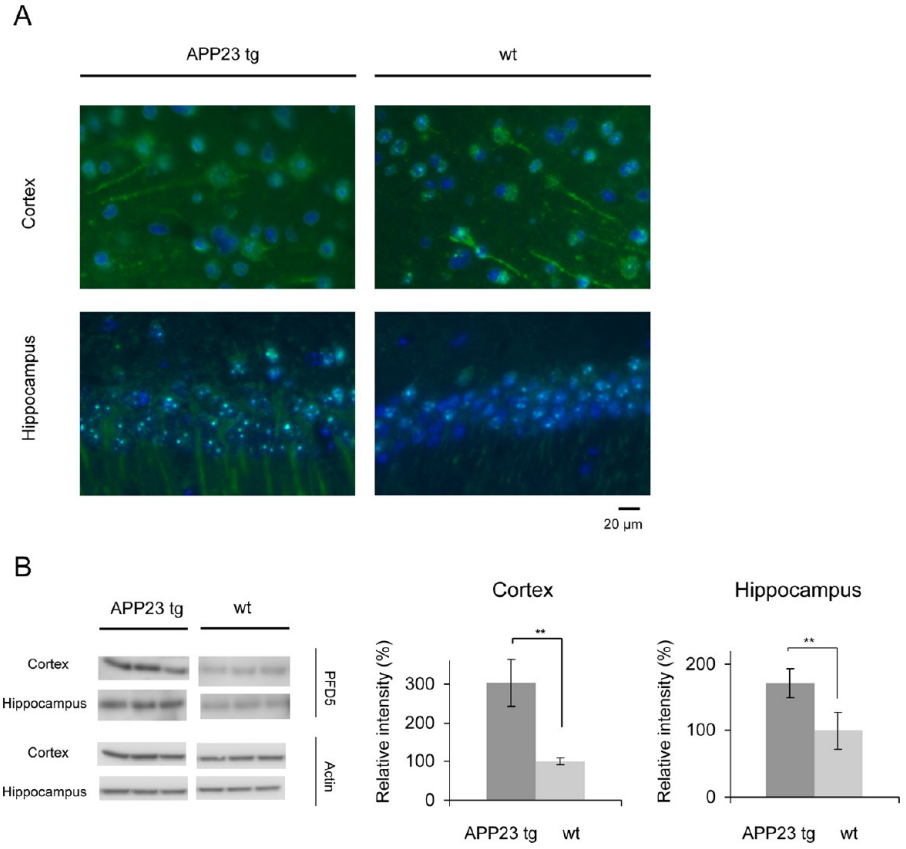


Figure 1. PFD expression in mouse brain. (A) Immunostaining of cortical and hippocampal brain sections from APP23 tg or wt mice with anti-hPFD5: green for PFD and blue for Hoechst. (B) Cortical and hippocampal brain homogenates prepared from APP23 tg or wt mice, analyzed by Western blotting using antibodies for PFD5 (n = 3 per genotype) and actin. Right panels show quantification of the Western blots confirming that expression of PFD is upregulated in APP23 tg mice brain. The intensities in wt mice were normalized as 100%. $^{**}p < 0.01$.

Proliferation Kit I (MTT) from Roche (Indianapolis, IN). PC12 cells (40000 cells/well in 80 μL of medium) were cultured on PDL-coated 96-well plates overnight. Aβ samples (20 μL), aliquoted from the incubated Aβ samples (with and without PFD) and diluted with PBS to the desired Aβ concentrations, were added to the wells and incubated for 16 h for PC12 cells or 24 h for primary cortical neurons. Because 0.5 μM Aβ fibrils exhibited cytotoxicity to PC12 cells (data not shown), the Aβ concentration in the cell culture medium was kept at 0.5 μM unless otherwise stated. For control samples, the same volume of PBS was added to the wells. For the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reaction and measurements, the adsorption values at 550 nm were determined using a Tecan (Männedorf, Switzerland) microplate reader. The viability for cells exposed to PBS was used as the 100% viability control.

Native PAGE and Western Blotting. The samples were diluted 1:1 with native PAGE sample buffer, applied to a 5 to 20% gradient Tris-glycine precast gel (Wako, Osaka, Japan), run at a constant current of 10 mA, and then transferred to a nitrocellulose membrane (0.22 μm) (Whatman, Kent, U.K.) at a current of 200 mA for 90 min. The membrane was blocked overnight at 4 °C with 5% skim milk in 0.01% TBST and probed with mouse monoclonal anti-Aβ (6E10, 1:2000, Covance, Princeton, NJ), mouse polyclonal anti-PhPFD[37] (1:2000), or anti-hPFD subunit 3 (hPFD3, 1:1000) (Santa Cruz Biotechnology) antibody, for 1 h at room temperature, followed by secondary horseradish peroxidase-conjugated anti-mouse or anti-goat IgGs (1:2000) (R&D Systems, Minneapolis, MN). Proteins were visualized using the ECL Plus blotting detection reagent (GE Healthcare) according to the manufacturer's instructions. Luminescence was detected in a LAS4000 mini Luminescent Image Analyzer (Fujifilm, Tokyo, Japan), with the Image Reader Las4000 software.

Transmission Electron Microscopy (TEM). One drop of sample was dispensed on a carbon-coated grid and allowed to air-dry. Samarium acetate was used to stain the sample. Samples were observed with an excitation voltage of 80 kV using a JEM-1230 transmission electron microscope (JEOL, Tokyo, Japan).

Immunoprecipitation. Aβ peptide (25 μM, 100 μL) was incubated with 5 μM hPFD. An incubated Aβ sample or hPFD alone samples were used as controls. The samples were diluted to a volume of 1 mL and incubated with 10 μL of anti-Aβ (4G8) overnight at 4 °C while being rotated. The samples were then incubated with 50 μL of Protein G-Sepharose beads (Amersham Biopharma Biotech, Uppsala, Sweden) for 7 h at room temperature. We pulled down all Aβ antibody-bound material with the beads by centrifuging the samples and separating the beads from the supernatant. The immunoprecipitates were washed three times with PBS and thereafter incubated with SDS sample buffer at 95 °C for 5 min. Eluted proteins were loaded on a 15% SDS−PAGE gel and analyzed via Western blotting using anti-hPFD3.

Dot Blot. The dot blot assay was performed as previously described.[41] Samples were incubated at 25 μM Aβ and 5 μM hPFD at 37 °C or at 50 μM Aβ and 50 μM PhPFD at 50 °C. For PFD concentration dependency experiments, 25 μM Aβ was incubated with various concentrations of hPFD (10, 5, 2.5,

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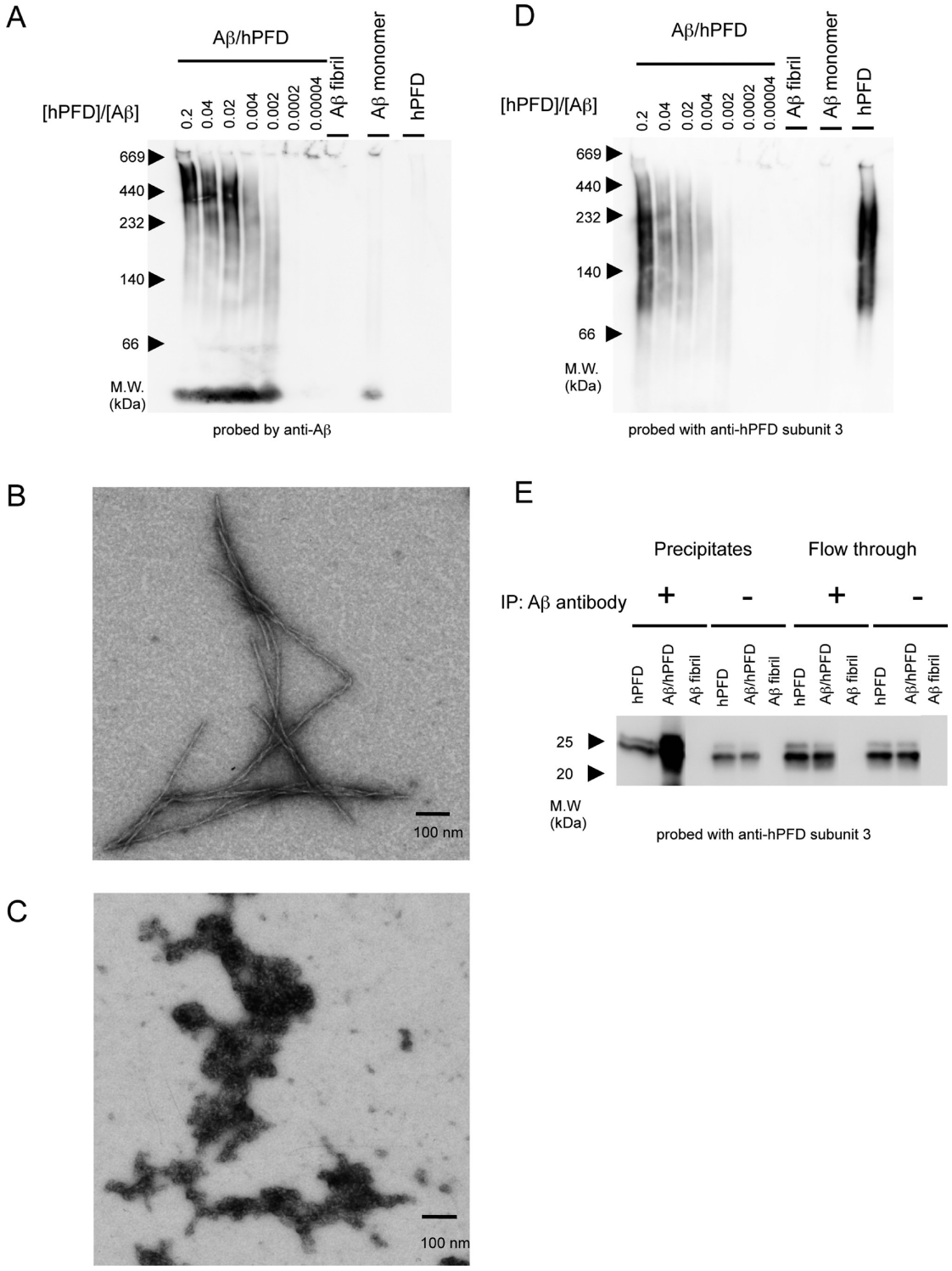

Figure 2. Formation of HMW Aβ oligomers by hPFD. (A) Native PAGE/Western blotting analysis of Aβ aggregates. Aβ samples (25 μM) incubated at 37 °C for 48 h in the absence (Aβ fibril) or presence of varied amounts of hPFD (Aβ/hPFD) were analyzed by native PAGE and probed using mouse monoclonal anti-Aβ (6E10). The ratio of hPFD concentration to Aβ concentration is shown. Unincubated Aβ monomers were used as a control. A HMW native marker kit (GE Healthcare) comprising thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), and albumin (66 kDa) was used as a molecular weight marker. (B and C) Morphology of insoluble Aβ fibrils formed in the absence of hPFD (B) or soluble Aβ aggregates formed in the presence of hPFD (C) monitored by TEM. The scale bars represents 100 nm. (D) Native PAGE/Western blotting analysis of Aβ samples incubated in the absence (Aβ fibril) or presence of varied amounts of hPFD (Aβ/hPFD) using anti-hPFD3. hPFD was used as a control at a concentration of 5 μM (corresponding to the highest concentration of hPFD incubated with Aβ). The same membrane used in panel A was used and reprobed. (E) Co-immunoprecipitation of hPFD and Aβ. Incubated samples were immunoprecipitated using anti-Aβ (4G8) and Protein G-Sepharose beads. The samples (precipitates and flow through) were then subjected to SDS−PAGE and Western blotting and probed with anti-hPFD3 to confirm interactions between the proteins. As control samples, hPFD alone and Aβ fibril samples were included in the analysis. Samples treated without anti-Aβ were also used as control samples.

dx.doi.org/10.1021/bi301705c | *Biochemistry* 2013, 52, 3532−3542

0.5, or 0.1 μM) and 50 μM Aβ was incubated with various concentrations of PhPFD (50, 10, 1, 0.2, or 0.01 μM) as described above. The co-incubated Aβ/PhPFD was diluted to an Aβ concentration of 25 μM prior to experiments. ADDL was prepared as described previously.[15] Aβ fibrils and an unincubated Aβ monomer sample were used as controls. The sample mixtures (4 μL) were thereafter spotted onto a nitrocellulose membrane. After being blocked with 5% skim milk and 0.01% TBST for 1 h at room temperature, the membrane was probed with the rabbit polyclonal anti-Aβ oligomer (A11, 1:500) (Biosource, Camarillo, CA), mouse monoclonal anti-Aβ oligomer (4D8, 1:500) (Agrisera, Vännäs, Sweden), or anti-Aβ (6E10, 1:2000) for 1 h at room temperature, followed by washing and probing with secondary antibodies as described above. The 6E10 antibody recognizes amino acid residues 1−16 of Aβ, both monomeric and aggregated forms, whereas 4D8 and A11 antibodies recognize certain Aβ oligomers. ImageJ was used to quantify the intensities of the dots.

ThT and ANS Fluorescence Assays. The structures of Aβ/hPFD and Aβ/PhPFD oligomers were assessed by ANS or ThT fluorescence assay. For ANS fluorescence measurements, Aβ samples (10 μM) were prepared in a PBS buffer containing 50 μM ANS (Sigma). The samples were excited at 370 nm (bandwidth of 10 nm), and the emission spectra were recorded at 470−580 nm. The fluorescence intensity of 50 μM ANS in a PBS solution was used for background subtraction for Aβ fibrils or Aβ monomers, and the fluorescence intensity for binding of ANS to hPFD or PhPFD alone was used as background subtraction for Aβ/hPFD or Aβ/PhPFD. For ThT fluorescence measurements, Aβ samples (5 μM) were prepared in a PBS buffer containing 50 μM ThT (Sigma). The samples were excited at 420 nm (bandwidth of 10 nm), and the emission spectra were recorded at 450−550 nm. The fluorescence intensity of 50 μM ThT in a PBS solution was used for background subtraction for Aβ fibrils or Aβ monomers, and the fluorescence intensity for binding of ThT to hPFD or PhPFD alone was used as background subtraction for Aβ/hPFD or Aβ/PhPFD. All measurements were performed using a Tecan microplate reader. The samples were assayed in triplicate, and the average of the three spectra is presented.

RESULTS

PFD Is Expressed in Mouse Brain. We analyzed brain sections of 6-month-old APP23 tg and wt mice by immunostaining (Figure 1A). Because deposits of Aβ are developed in the cortex and hippocampus of APP23 tg mice,[38] we examined brain sections containing these parts. As shown in the figure, PFD is expressed in the brain in both APP23 tg and wt mice. The expression level of PFD in brains of APP23 tg or wt mice was estimated by analyzing cortical and hippocampal brain homogenate by Western blotting with antibodies directed to PFD subunit 5 (Figure 1B). As shown in the figure, expression of PFD was confirmed in the cortex and hippocampus. Importantly, the level of PFD expression was elevated in both cortex and hippocampus of the APP23 tg mice (Figure 1B), suggesting the possible biological relevance of PFD in AD.

Formation of Soluble Aβ Oligomers in the Presence of hPFD. The effect of hPFD on the aggregation of the Aβ(1−42) peptide was examined *in vitro* with native PAGE and TEM analysis. We previously reported that Aβ fibrillation was inhibited in the presence of archaeal PhPFD.[37] In this work, we examined the fibrillation behavior of Aβ in the presence of hPFD. Figure 2A shows native PAGE/Western blotting analysis of Aβ samples incubated with varied amounts of hPFD. Co-incubation of Aβ and hPFD (Aβ/hPFD) resulted in formation of large soluble Aβ oligomers in a molecular weight distribution between 300 and 600 kDa (Figure 2A), while Aβ incubated in the absence of hPFD under the same conditions did not yield HMW oligomers, indicating that large, insoluble fibrils had been formed that could not enter the gel; these fibrils were confirmed by TEM (Figure 2B). Formation of soluble oligomers was also confirmed with SDS−PAGE/Western blotting analysis (data not shown). Next we investigated the necessary amount of hPFD required to obtain soluble Aβ oligomers and found that an hPFD:Aβ ratio as low as 0.02 (1:50) was sufficient to induce the formation of soluble Aβ oligomers. Even at hPFD:Aβ ratios as low as 0.002 (1:500), soluble Aβ species, including Aβ monomers, were detectable. At lower hPFD concentrations, no soluble Aβ species were observed, which suggests insoluble Aβ fibril formation. These data indicate that substoichiometric amounts of hPFD relative to Aβ are sufficient to observe the antifibrillation phenomenon. The TEM data support the native PAGE/Western blotting data, as large oligomers could be seen in the samples where hPFD was present during incubation (Figure 2C). In soluble Aβ/hPFD, short, prefibrillar structures could also be observed, along with smaller fragments. On the basis of the finding that hPFD inhibited Aβ fibril formation, we studied formation of the complex between hPFD and Aβ by using Western blotting and immunoprecipitation. Figure 2D shows Aβ/hPFD that have been transferred to a nitrocellulose membrane and probed with anti-hPFD, derived from a native gel used in Figure 2A. hPFD alone appeared on the gel within the molecular weight range of ∼100−400 kDa. Co-incubated Aβ/hPFD gave an hPFD signal within the molecular weight range of ∼100−500 kDa with main protein fractions found between 200 and 300 kDa. Because most of the Aβ was detected in the molecular weight range of 300−500 kDa (Figure 2A), most of the hPFD is most likely not in complex with the Aβ oligomers.

To further examine the possible formation of a complex, co-incubated Aβ/hPFD were immunoprecipitated using anti-Aβ (4G8) and Protein G-Sepharose beads and detected by Western blotting using anti-hPFD (Figure 2E). As shown in the figure, a significant amount of hPFD could be detected in the precipitate, supporting a direct interaction between hPFD and Aβ. For the control experiments, the samples were precipitated in the absence of anti-Aβ. The faint bands observed in the control experiments are likely to be due to unspecific binding of hPFD to the Sepharose beads (however, not to anti-Aβ, because the intensity of the band of hPFD incubated with anti-Aβ was similar to the intensity of the band of hPFD incubated without anti-Aβ). Taken together, although these results propose a direct interaction between hPFD and Aβ for oligomer formation, the binding is likely to be transient and/or weak.

Toxicity of Aβ/hPFD Oligomers to PC12 and Primary Neuron Cells. Accumulated evidence has suggested that soluble Aβ oligomers are toxic to neurons in AD brains.[4−9,11,37] To investigate the cytotoxicity of the Aβ oligomers, we added Aβ oligomers to cultured rat pheochromocytoma PC12 cells, or primary neurons. The cytotoxicity of the Aβ oligomers formed in the presence of hPFD to PC12 cells is shown in Figure 3A. Intriguingly, the Aβ oligomers formed in the presence of hPFD resulted in a cell viability higher than that for Aβ fibrils alone.

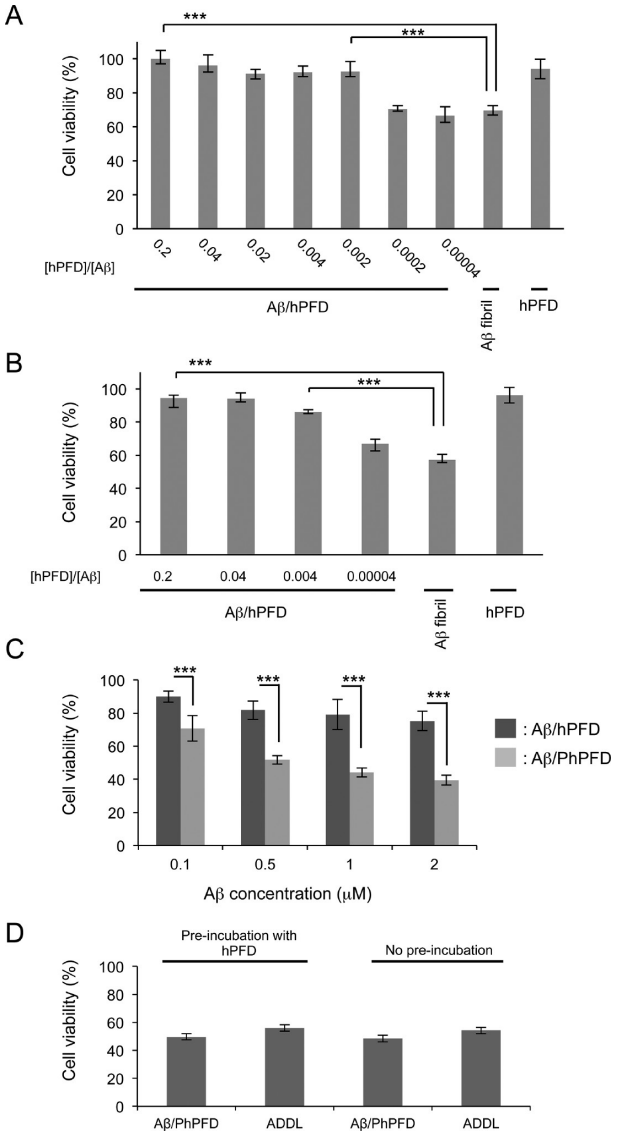

Figure 3. Reduced cytotoxicity of Aβ oligomers formed by hPFD. (A and B) Cytotoxicity assays of Aβ oligomers formed in the presence of hPFD with PC12 cells (A) and differentiated PC12 cells with NGF (B) using the MTT assay. The ratio of hPFD concentration to Aβ concentration (25 μM) during incubation is shown. The Aβ concentration in the cell culture medium was 0.5 μM. Aβ fibrils and hPFD were used as control samples. Data are shown as means ± the standard deviation (SD) of three experiments. ***p < 0.001. (C) Cytotoxicity assays of Aβ oligomers formed with hPFD (Aβ/hPFD) or PhPFD (Aβ/PhPFD) samples against primary cortical neurons using the MTT assay. The Aβ samples were incubated with the cells for 24 h at the indicated concentrations prior to viability measurements. ***p < 0.001. (D) Viability of PC12 cells exposed to toxic Aβ oligomers after preincubation with hPFD. hPFD (1 μM) was added to the cell medium 4 h prior to the addition of 0.5 μM toxic Aβ oligomers (Aβ/PhPFD or ADDL).

To investigate the concentration of hPFD needed to inhibit Aβ cytotoxicity, we compared the toxicity of samples incubated at various hPFD concentrations (Figure 3A). We observed that a molar concentration of hPFD as much as 500-fold lower than the concentration of Aβ ([hPFD]/[Aβ] = 0.002) significantly reduced the cytotoxicity. The ratios of hPFD to Aβ that reduce cytotoxicity are similar to those that prevented *in vitro* fibrillation (Figure 2A). It should be noted that the cell toxicity was similar for NGF differentiated PC12 cells (Figure 3B). However, the reduced toxicity was unexpected because our previous study showed that Aβ oligomers formed by co-incubation of Aβ and archaeal PhPFD (Aβ/PhPFD) were more cytotoxic than Aβ fibrils.[37] Thus, we also examined the cytotoxicity of the Aβ oligomers using primary neurons, by comparing the viability of cells that had been exposed to either Aβ/hPFD or Aβ/PhPFD at different concentrations (Figure 3C). As shown in the figure, primary neurons exposed to hPFD-formed Aβ oligomers were significantly more viable than cells exposed to PhPFD-formed Aβ oligomers.

We then addressed whether the reduced toxicity of the Aβ oligomers formed in the presence of hPFD could be due to the ability of hPFD itself to protect the cells against cell death. To examine this, we preincubated PC12 cells with 1 μM hPFD for 4 h, before addition of 0.5 μM toxic Aβ oligomers (ADDL or Aβ/PhPFD), followed by viability measurements with the MTT assay (Figure 3D). Our results show clearly that the cytotoxicity was preserved, even with hPFD in the solution, which indicates that hPFD itself does not prevent the toxic effect of preformed toxic oligomers.

Difference between Aβ Oligomers Formed by hPFD and PhPFD. Our study revealed differences in cytotoxicity between Aβ oligomers formed by eukaryotic hPFD and archaeal PhPFD. To compare these Aβ oligomers of different toxicities, co-incubated Aβ/hPFD or Aβ/PhPFD were subjected to native PAGE followed by Western blotting using anti-Aβ (Figure 4A). Incubation temperatures and concentrations appropriate for obtaining effective Aβ oligomerization in the presence of each chaperone were applied. Formed Aβ oligomers appeared to be of similar size (HMW soluble oligomers within the molecular weight range of 200−500 kDa) regardless of which chaperone we used (Figure 4A). Then we hypothesized that the HMW Aβ oligomers formed by co-incubation with hPFD or PhPFD might differ in shape and/or surface structure with different parts of the Aβ sequence exposed, leading to different toxic properties. Comparing TEM data of the Aβ/hPFD oligomers (Figure 2C) and the Aβ/PhPFD oligomers,[37] we could not observe significant differences between the oligomers, which both consisted of large oligomers and prefibrillar aggregates. To further investigate possible differences in structures, we examined the antibody recognition properties of the Aβ oligomers using various anti-Aβ antibodies. The hPFD- or PhPFD-formed Aβ oligomer samples were spotted on nitrocellulose membranes and probed with 6E10, 4D8, or A11 antibodies, and the intensities of the signals were compared (Figure 4B). We obtained different intensities of signals for the different antibodies reacting with the same concentration of different Aβ oligomeric species, Aβ monomers, or Aβ fibrils. The 4D8 antibody bound more strongly to Aβ/PhPFD than Aβ/hPFD. On the other hand, the binding of A11 to Aβ/hPFD was stronger than that to Aβ/PhPFD (Figure 4B). The intensity of the Aβ/PhPFD dot was approximately 60% of the intensity of the Aβ/hPFD dot for the A11 antibody. To further confirm the difference in antibody recognition, Aβ/hPFD and Aβ/PhPFD incubated at various concentrations of hPFD or PhPFD were prepared and analyzed with a dot blot assay using the A11 antibody (Figure 4C). As shown in the figure, the antibody signal intensities increased in a dose-dependent manner when Aβ was incubated with PFDs at higher concentrations, and the A11 antibody showed stronger affinity for Aβ/hPFD than for Aβ/PhPFD at different

dx.doi.org/10.1021/bi301705c | *Biochemistry* 2013, 52, 3532−3542

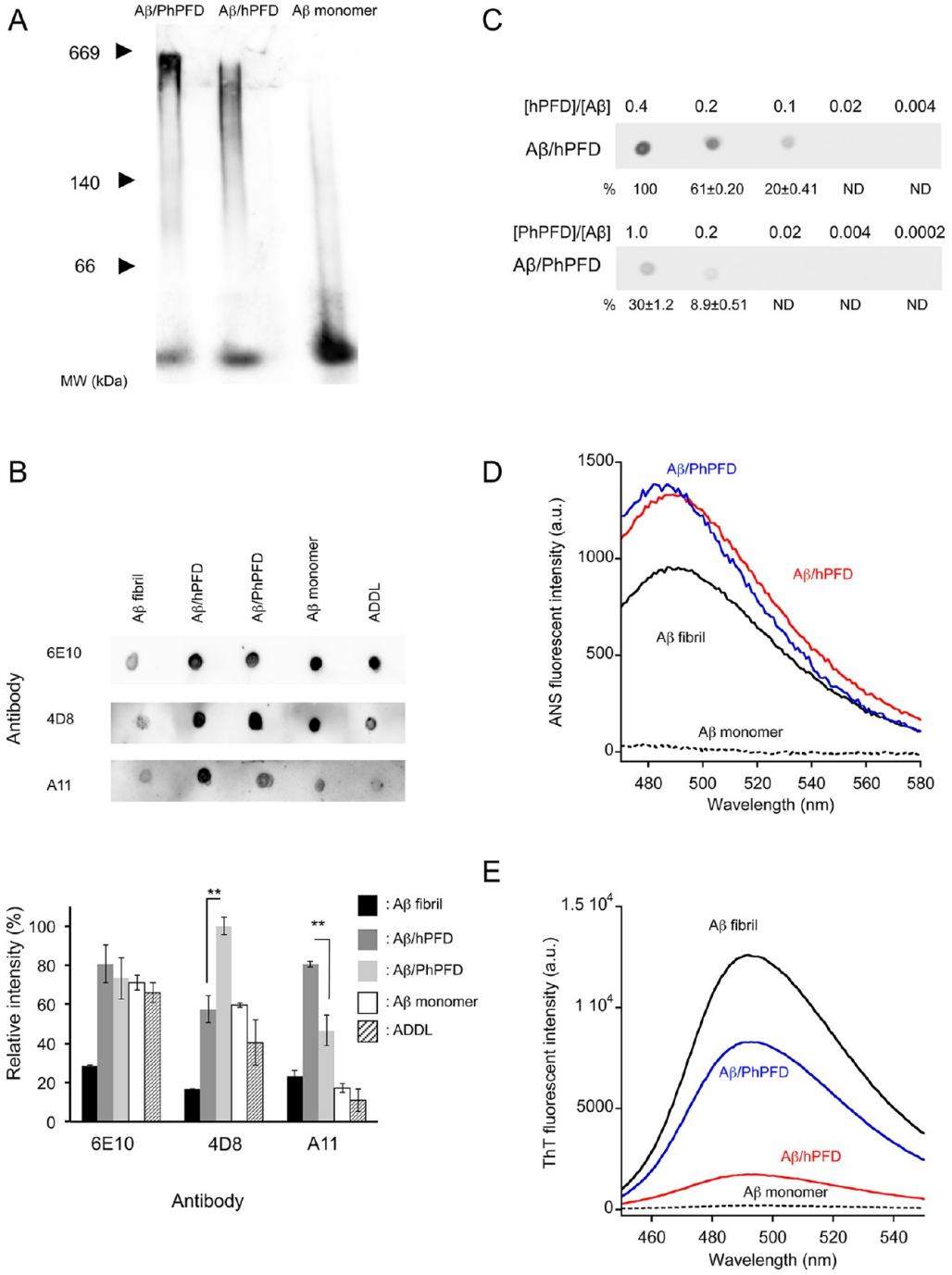

Figure 4. Comparison between Aβ oligomers formed by hPFD and PhPFD. (A) Native PAGE/Western blotting analysis of Aβ oligomers formed in the presence of PhPFD (Aβ/PhPFD) and hPFD (Aβ/hPFD). Incubated samples were analyzed by native PAGE and probed using mouse monoclonal anti-Aβ (6E10). The Aβ monomer was used as a control. (B) Dot blot assay of Aβ oligomers formed in the presence of hPFD (Aβ/hPFD) or PhPFD (Aβ/hPFD) using various Aβ antibodies as indicated. Aβ fibril, Aβ monomer, or ADDL was used for comparison. Samples were spotted onto nitrocellulose membranes and probed with the 6E10, 4D8, or A11 anti-Aβ antibody. The intensity of each spot was quantified with ImageJ and compared, confirming the different affinities of the different Aβ oligomers for the different antibodies. The intensity in Aβ/PhPFD with the 4D8 antibody was normalized as 100%. Data are shown as means ± SD of two spots. $^{**}p < 0.01$. (C) Dot blot of Aβ oligomers formed in the presence of various concentrations of hPFD or PhPFD. Samples were spotted onto a nitrocellulose membrane probed with the A11 antibody. ImageJ was used for quantification. The intensity in Aβ/hPFD ([hPFD]/[Aβ] = 0.4) was normalized as 100%. Data are shown as means ± SD of two spots. (D) ANS fluorescence spectra comparing the Aβ fibril (black), Aβ/PhPFD (blue), Aβ/hPFD (red), and Aβ monomer (dotted). Samples were prepared in triplicate at 10 μM Aβ and 50 μM ANS in a volume of 100 μL. ANS fluorescence with hPFD or PhPFD alone was used as a background and subtracted from the Aβ/hPFD or Aβ/PhPFD, respectively. The samples were excited at 370 nm. Emission was measured from 470 to 580 nm. (E) ThT fluorescence spectra comparing the Aβ fibril (black), Aβ/PhPFD (blue), Aβ/hPFD (red), and Aβ monomer (dotted). Samples were prepared in triplicate at 5 μM Aβ and 50 μM ThT in a volume of 100 μL. As described above, the ThT fluorescence with hPFD or PhPFD alone was used as background subtraction for Aβ/hPFD or Aβ/PhPFD. The samples were excited at 420 nm. Emission was measured from 450 to 550 nm.

dx.doi.org/10.1021/bi301705c | *Biochemistry* 2013, 52, 3532−3542

concentrations, which also supports different antibody recognition properties between these two oligomers.

The structural properties of Aβ/hPFD and Aβ/PhPFD oligomers were further examined by the hydrophobicity probe, ANS, and the amyloid probe, thioflavin T (ThT). As shown in Figure 4D, binding of ANS to Aβ/hPFD or Aβ/PhPFD was similar, which indicates that the surface hydrophobicity of the compared Aβ oligomers is similar. On the contrary, in the case of ThT binding, a significant difference in ThT binding was observed between Aβ oligomers formed in the presence of either hPFD or PhPFD (Figure 4E). It was previously shown that Aβ/PhPFD gave a weaker ThT fluorescent signal than Aβ fibrils.[37] Here, we compared the ThT binding properties for Aβ/PhPFD and Aβ/hPFD and found that Aβ/hPFD gave an even weaker ThT fluorescent signal than Aβ/PhPFD, which suggests that the amount of accumulated β-sheets of Aβ/hPFD is smaller than of Aβ/PhPFD, or that the β-sheet stacking manner is different. Taken together, these data suggest that difference in toxicity between Aβ oligomers formed by human and archaeal PFD might be correlated with differences in protein conformation, such as exposed amino acid residues, geometrical features, and β-sheet packing.

DISCUSSION

Soluble oligomers of Aβ are considered toxic species that contribute to AD pathology.[4−11] For example, Aβ oligomers can block hippocampal long-term potentiation *in vivo*[42] and trigger synaptic loss leading to cognitive impairment.[43] The structures and sizes of soluble Aβ oligomers vary widely, and which oligomeric species represents the toxic form(s) remains unclear. Protein misfolding and aggregation are very complex processes; aside from the size of Aβ oligomers potentially correlating with its toxic properties, the surface structure, shape, and composition of the oligomers likely represent important parameters of toxicity. The Aβ aggregation pathway might also be of importance for its toxicity, and environmental factors such as chaperones or other components of the cellular homeostasis machinery might play important roles in this pathway.[11,26,44,45]

Molecular chaperones are known to prevent protein aggregation and to function to protect against toxicity of misfolded proteins.[26,44−46] There are studies describing how molecular chaperones can prevent toxicity of Aβ, or other proteins that are toxic to cells.[27,47−51] However, there are also contradictory reports suggesting that chaperones might trap the growing amyloids in a toxic state.[37,52,53]

In this study, we confirmed the presence of PFD in the cortex and hippocampus of APP23 tg or wt mice, the same regions of the brain that are strongly affected in AD. We also investigated the effect of hPFD on Aβ aggregation using recombinant hPFD and demonstrated for the first time that Aβ fibrillation was inhibited in the presence of hPFD and large, soluble oligomers were formed. Even at substoichiometric hPFD:Aβ ratios down to 1:500, hPFD had an effect on Aβ fibrillation (Figure 2A). One of the important cellular roles of a molecular chaperone is to bind and release its substrate in a repetitive cycle, until the target protein has been correctly folded,[22,24,54] potentially explaining how comparatively low levels of chaperone complexes can affect the folding of a molar excess of a target substrate. This is also consistent with our results suggesting that the interaction between hPFD and Aβ is likely to be transient and/or weak (Figure 2).

Importantly, we found that Aβ oligomers formed in the presence of hPFD were less toxic to cultured cells and primary neurons, which contradicts our previous finding of toxic Aβ oligomers formed in the presence of archaeal PhPFD.[37] The sizes of these oligomers appeared to be similar at least by native gel analysis. However, we found that both antibody recognition and ThT binding were different, which suggests that there are differences in the molecular surfaces and conformations of these oligomers. Previous studies have shown that the substrate binding mechanism differs between eukaryotic and archaeal PFD.[34,35] Thus, it is plausible that different substrate binding between hPFD and PhPFD might lead to differences in the modulation of oligomerization, and ultimately different physical and chemical attributes of the surface structure.

It has been shown that the structures of Aβ oligomers are related to their toxic properties. Takamura et al. reported that recognition of Aβ HMW oligomers with newly generated monoclonal antibodies could be correlated with their toxicity.[18] In a recent paper by Sinha et al., the authors reported that lysine specific molecular compounds inhibited fibrillation of amyloidogenic proteins and induced nontoxic oligomer formation.[55] These nontoxic oligomers had a lower affinity for the A11 antibody than toxic oligomers. In another study, the A11 antibody was found to recognize soluble oligomers of various proteins.[41,56] It was also shown that the two oligomers with different toxicities showed differences in their recognition by Aβ antibodies.[57] In another study by Noguchi et al., toxic oligomers were not recognized by A11 oligomers, but by another oligomer specific antibody.[58] These studies suggest that antibody recognition of Aβ oligomers is related to their toxic properties, which is consistent with our results. Previous studies showed that oligomer toxicity could be mediated via cell surface receptors.[11,59,60] Thus, it is plausible that receptor recognition could be different between the PhPFD- and hPFD-formed oligomers. Another hypothesis is that the interactions between the oligomers and the cell membranes are crucial for the toxicity, and that toxic oligomers can disturb the monolayers in the cell membrane and cause cell death.[61] Thus, it is also plausible that interactions with cell membranes might be different between PhPFD- and hPFD-formed oligomers.

Our results using structural probes such as ANS and ThT strengthen the idea that different structures of protein aggregates decide their toxic properties. ANS is a molecule that recognizes hydrophobic sites in proteins. Studying the binding affinity of ANS for different protein structures therefore gives a hint about the amount of exposed hydrophobic sites in the structures. We did not obtain significantly different ANS fluorescence results while comparing Aβ/hPFD and Aβ/PhPFD, which indicates that the surface hydrophobicities of the different oligomers are similar. Interestingly, however, the ThT fluorescence values showed a clear difference. ThT is believed to recognize and bind to the "cross β structure", which is a common motif in protein fibrils and aggregates.[62] Therefore, our data show that the less toxic Aβ oligomer might contain fewer cross β motifs. It was recently described that toxic fibrillar oligomers of Aβ are rich in cross β structure,[63] which is consistent with our ThT binding and toxicity data. When these studies are put together, it is plausible that the inner and outer structure of Aβ oligomers might be crucial in Aβ-mediated toxicity, such as the composition of exposed amino acid residues and protein packing.

PFD is known as a cytosolic protein, with a crucial function in protein folding.[22] However, it might be found in the extracellular space. It has been shown that tau and other intracellular components can be found in the extracellular space

upon neuron death, and subsequently in the cerebrospinal fluid, for example, in AD.[64−66] Thus, it is possible that PFD also could be released into the extracellular space. In addition, it has recently become obvious that Aβ is produced intracellularly (see review 9, for example). Recent findings also support the idea that Aβ is present within the cytosolic compartment. For example, intracellular accumulation of Aβ is linked to cytosolic proteasome inhibition, and proteasome inhibition leads to higher Aβ levels.[67−69] In this study, we have shown that PFD is expressed in cortical neurons and is upregulated in APP23 tg mice, supporting a putative connection of hPFD to AD *in vivo*. This is consistent with a recent genomewide expression study of AD patients, which showed that PFD and CCT genes are upregulated in AD.[70,71] Thus, it is possible that hPFD could modulate the folded state of a subpopulation of intracellular Aβ to exert protective effects on cells.

The data presented in this work provide the first description of a potential molecular basis for hPFD in modulating Aβ aggregation. Our findings also contribute to the understanding of the diversity of Aβ oligomerization and its subsequent toxicity and will stimulate other studies involving the relationship between molecular chaperones and their influence on protein misfolding disorders such as AD.

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Funding

We are grateful for financial support from RIKEN (K.M.S., T.Z., and M.M.), the Ministry of Education, Science, Sports, Culture and Technology of Japan (MEXT) (T.Z. and M.M.), and the Canadian Institutes of Health Research (CIHR; Grant MOP-84523 to M.R.L.). K.M.S. is a Foreign Postdoctoral Researcher of RIKEN. P.C.S. acknowledges scholarships from the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Michael Smith Foundation for Health Research (MSFHR). M.R.L. is the recipient of a senior scholar award from the MSFHR.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS

AD, Alzheimer's disease; Aβ, amyloid-β; ADDLs, Aβ-derived diffusible ligands; PFD, prefoldin; CCT, cytosolic chaperonin containing TCP-1; PhPFD, PFD from *P. horikoshii*; TEM, transmission electron microscopy; Aβ/hPFD, Aβ sample(s) incubated with hPFD; Aβ/PhPFD, Aβ sample(s) incubated with PhPFD; ThT, thioflavin T.

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