Soluble Prion Protein Inhibits Amyloid-β (Aβ) Fibrillization and Toxicity*

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Background: Prion protein was found to interact with Aβ, but the consequences of this interaction are largely unknown.

Results: Prion protein and its N-terminal fragment inhibit Aβ1–42 amyloidogenesis and cytotoxicity.

Conclusion: Soluble prion protein is a potent inhibitor of Aβ1–42 assembly into toxic oligomers.

Significance: The results have important implications for understanding the pathogenesis of AD and for the development of novel therapeutic strategies.

The pathogenesis of Alzheimer disease appears to be strongly linked to the aggregation of amyloid-β (Aβ) peptide and, especially, formation of soluble Aβ1–42 oligomers. It was recently demonstrated that the cellular prion protein, PrPC, binds with high affinity to these oligomers, acting as a putative receptor that mediates at least some of their neurotoxic effects. Here we show that the soluble (i.e. glycosylphosphatidylinositol anchor-free) prion protein and its N-terminal fragment have a strong effect on the aggregation pathway of Aβ1–42, inhibiting its assembly into amyloid fibrils. Furthermore, the prion protein prevents formation of spherical oligomers that normally occur during Aβ fibrillogenesis, acting as a potent inhibitor of Aβ1–42 toxicity as assessed in experiments with neuronal cell culture. These findings may provide a molecular level foundation to explain the reported protective action of the physiologically released N-terminal N1 fragment of PrPc against Aβ neurotoxicity. They also suggest a novel approach to pharmacological intervention in Alzheimer disease.

Alzheimer disease (AD) is associated with the accumulation of extracellular amyloid plaques, the main component of which is the 40–42-residue amyloid-β (Aβ) peptide (1, 2). This peptide is derived from the proteolytic processing, by β- and γ-secretases, of a large membrane glycoprotein known as amyloid precursor protein (1–3). Although for many years the main focus of research has been on mature Aβ amyloid fibrils, more recent studies point to a potentially crucial role in the disease pathogenesis of smaller, nonfibrillar Aβ aggregates, often referred to as soluble oligomers. Indeed, the latter species were shown to act as potent neurotoxins in vivo and in vitro (4–6), and their presence appears to correlate with disease progression in patients with AD and animal models of the disease (5, 7, 8). However, the mechanisms by which Aβ oligomers exert the neurotoxic effects that result in cognitive impairment remain unclear.

Prion protein (PrPc) is a glycoprotein tethered to the plasma membrane via the glycosylphosphatidylinositol anchor. Although this protein is best known for its role in the pathogenesis of transmissible spongiform encephalopathies (9), a series of recent studies points to a potentially important link between PrPc and AD (10–16). Especially intriguing in this regard are the reports that PrPc may act as a cell surface receptor that binds Aβ oligomers, mediating effects such as Aβ-induced impairment of synaptic plasticity (11, 17) and cognition (10) as well as neuronal cell death (18). Although some of these findings have been recently challenged (19, 20), there appears to be a consensus that prion protein binds with high affinity to Aβ oligomers, whereas no such binding occurs to Aβ monomers or mature amyloid fibrils (11, 19, 21). Apart from the proposed role as a mediator of Aβ synaptotoxicity, this high affinity interaction may have other consequences of relevance to understanding the mechanism of Aβ aggregation as well as its neurotoxic effects.

Here we report that the glycosylphosphatidylinositol anchor-free recombinant prion protein and its N-terminal fragment have a strong effect on the aggregation pathway of Aβ1–42, acting as a potent inhibitor of fibrillization and preventing formation of toxic oligomers that normally occur at early stages of this reaction. These findings have potentially important implications for understanding the pathogenic process in AD as well as for the development of novel therapeutic strategies.

EXPERIMENTAL PROCEDURES

Preparation of Prion Protein and Its Fragments—Plasmids encoding full-length human PrP (PrP23–231) and its truncated variants PrP23–144, PrP90–231, and PrP122–231 were previously described (22). Bacterial expression and protein purification of recombinant PrP23–231 and its fragments were performed as described previously (22, 23). Protein concentration was determined by measuring absorbance at 276 nm using the appropriate extinction coefficients. Amyloid fibrils of PrP23–231 were generated as described previously (24).

Short synthetic peptides corresponding to human PrP fragments encompassing residues 23–32 (PrP23–32) and residues 94–110 (PrP94–110) were synthesized commercially by EZBiolab (Westfield, IN). PrP23–32 contained additional N-terminal glycine residue, and both peptides were biotiny-
lated at their N termini. The peptides were better than 95% pure as assessed by HPLC and mass spectrometry analyses.

Preparation of Aβ Peptides—Human Aβ1–42 was purchased from American Peptide Co. (Sunnyvale, CA). Lyophilized peptide was solubilized in hexafluoro-2-propanol, divided into aliquots and, after solvent evaporation, stored at −80 °C. To remove any residual aggregates, immediately before use, the peptide was dissolved in 10 mM NaOH to a concentration of 400 μM and subjected to 10 cycles of 10-s sonication on ice. Subsequently, 1 volume of the solution was combined with 3 volumes of 10 mM sodium phosphate, pH 7.4, yielding a peptide concentration of 100 μM.

Measurements of Thioflavin T (ThT) Fluorescence—Aβ peptides (20 μM) were incubated at 37 °C in 50 mM sodium phosphate, pH 7.4, containing 10 μM ThT, in 96-well plates. The plates were placed in BioTek FLx800 plate reader (BioTek Instruments, Winooski, VT), and fluorescence measurements were carried out every 20 min at 485 nm upon excitation at 440 nm. Before each reading, the plate was subject to shaking for 10 s.

Atomic Force Microscopy (AFM)—Samples to be analyzed by AFM were withdrawn at different stages of reaction from the 96-well plates and placed on freshly cleaved mica substrate for 3 min. After rinsing with 150 μl of deionized water and drying with stream of nitrogen, the samples were imaged in a tapping mode using a MultiMode atomic force microscope (Digital Instruments, Santa Barbara, CA) equipped with a NanoScope IV controller.

Cell Culture and Toxicity Measurements—Human neuroblastoma M17 cells were cultured in DMEM/F12 medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cultures were kept at 37 °C in a humidified incubator with 5% CO2. The cells were then plated at a density of 2 × 104/well on a 96-well plate and cultured for an additional 48 h. Before treatment with Aβ, the medium was replaced with DMEM/F12 without phenol red and serum. Preparations of Aβ1–42 (20 μM) were incubated as described for fluorescence measurements, with the exception that no ThT was present in the reaction mixture. Immediately before use, aliquots were withdrawn from the plates and diluted 4-fold into the cell culture medium. After a 48-h treatment with Aβ preparations, cell viability was assessed using lactate dehydrogenase assay (Takara Bio Inc., Shiga, Japan) according to the manufacturer’s instructions. Absorbance at 490 nm was measured in the microplate reader (SpectraMax 340, Molecular Devices, Sunnyvale, CA) and normalized against the medium without phenol red and serum. Data are expressed as the ratio of the absorbance for Aβ-treated and control (untreated) cells.

RESULTS AND DISCUSSION

Previous studies have shown that membrane-tethered PrP-C as well as the glycosphatidylinositol anchor-free recombinant PrP bind with high affinity to Aβ1–42 oligomers (11, 20, 21). Because morphologically similar oligomeric species are often observed as intermediates at early stages of Aβ fibrillation (5, 25–27), this raises important questions regarding the effect of PrP on the aggregation pathway of the Aβ peptide. To probe this issue, preparations of monomeric Aβ peptide (dis-aggregated as described under “Experimental Procedures”) were mixed with increasing concentrations of full-length recombinant human prion protein (PrP23–231), and the fibrillation/aggregation reaction was monitored by ThT fluorescence and AFM. Under the present experimental conditions (with intermittent shaking before each fluorescence reading), the reaction for Aβ1–42 alone is characterized by a lag phase of ∼2–2.5 h followed by a rapid increase in ThT fluorescence that reaches saturation after ∼7 h (Fig. 1A). Consistent with previous studies (26, 27), during the lag phase, AFM reveals formation of largely spherical oligomers with variable size (the height
in AFM images of ~2–5 nm) (Fig. 1B). These oligomers disappear with time and are gradually replaced by long amyloid fibrils (Fig. 1, C and D).

PrP23–231 effectively inhibits formation of ThT-positive structures that are observed for Aβ1–42 alone. Remarkably, this inhibitory effect is already significant at a protein concentration as low as 0.1 μM (or 1:200 PrP:Aβ monomer molar ratio), in which case the lag phase is extended to ~5 h. This effect gradually increases with increasing concentration of PrP23–231. In the presence of 2 μM protein, no ThT-positive structures could be detected even after a 24-h incubation. The reaction mixtures containing Aβ1–42 and PrP23–231 (PrP:Aβ molar ratio of 1:20) were analyzed by AFM, revealing formation of structures that are morphologically different as compared with those observed for the peptide alone. In particular, spherical oligomers that are abundant after 2 h (i.e. near the end of the lag phase) in experiments with Aβ1–42 alone (Fig. 1B) appear to be almost completely absent in samples containing PrP23–231. Instead, the latter samples show much smaller particles of irregular shape, with the height in AFM images below 1 nm (Fig. 1E). Importantly, very few spherical 2–5-nm oligomers characteristic for early stages of the reaction with Aβ1–42 alone (and no fibrillar structures) are detectable even after a 15-h incubation (Fig. 1, F and G), a time at which Aβ1–42 alone shows an extensive network of amyloid fibrils and residual spherical oligomers (Fig. 1, C and D). However, after such long incubation times, large amorphous aggregates are also present in Aβ1–42/PrP samples (Fig. 1F). In contrast to monomeric prion protein, amyloid fibrils generated from PrP23–231 did not affect the kinetics of Aβ1–42 fibrillation, even when used at high concentration (PrP23–231:Aβ1–42 molar ratio of 1:5) and fragmented by sonication into relatively small particles (data not shown).

To identify specific region(s) within the PrP molecule that are crucial for the inhibition of Aβ1–42 fibrillation, we employed a panel of deletion variants of the prion protein as well as synthetic peptides corresponding to fragments thereof (Fig. 2A). Importantly, the C-terminally truncated protein PrP23–144 was found to be as effective an inhibitor as the full-length PrP23–231 (Fig. 2B), whereas the N-terminally truncated protein PrP122–231 showed no inhibitory activity (Fig. 2C). Furthermore, the variant lacking residues 23–89 (PrP90–231) was partially active, showing comparable inhibitory effects on Aβ1–42 fibrillation at ~10-fold higher concentrations than the full-length PrP23–231 or PrP23–144 (Fig. 2D). Under the same experimental conditions, none of the PrP fragments alone gave any ThT response (data not shown). Collectively, these findings are consistent with previously published data indicating that two regions within the N-terminal part of PrP, one encompassing residues ~95–110 and one consisting of residues 23–27, are important determinants of high affinity binding to Aβ oligomers (21), although there is some controversy regarding the role of the N-terminal region (17). However, in contrast to the partial activity of PrP90–231, short peptides PrP94–110 or PrP23–32, even when used at high concentrations (100 μM), showed very little inhibitory effect (Fig. 2E). Thus, it appears that longer polypeptide chains containing these binding sites are required to inhibit fibrillation of Aβ1–42.

Broadly defined oligomeric species rather than mature fibrils are believed to be the most neurotoxic assemblies of Aβ peptide (4–6, 26). Consistent with this view, high toxicity was reported for oligomers formed at early stages (i.e. during the lag phase) of the Aβ1–42 fibrillation reaction (25). Given the dramatic effect of the prion protein on the aggregation/fibrillation pathway of Aβ1–42, we tested the toxicity of the peptide complexes with PrP23–231. To this end, the effect of Aβ1–42 preparations preincubated with and without PrP23–231 on viability of M17 neuroblastoma cells was assessed by measurements of lactate dehydrogenase release into the culture medium, an assay frequently used in studies of Aβ toxicity. Consistent with a previous study (25), the cells treated with Aβ1–42 preparations withdrawn shortly after the beginning of incubation and after 10 or more hours of incubation (i.e. at the stage corresponding to dominant presence of amyloid fibrils) showed relatively little toxicity. However, a substantial toxic effect was observed after 2 h of incubation (Fig. 3), i.e. at the time point corresponding to a large concentration of the spherical oligomers (Fig. 1B). Importantly, this toxic effect was not found for the preparations of Aβ1–42 preincubated for the same period of time in the presence of the prion protein. Furthermore, no toxicity beyond the level observed for PrP23–231 alone was observed for the latter preparations preincubated for 15 h. Thus, these data clearly indicate that the recombinant prion protein inhibits formation of cytotoxic assemblies of Aβ1–42.
This finding may explain the recent observation that the expression of secreted (i.e. glycosylphosphatidylinositol anchor-free) PrP suppressed the impairment of long-term potentiation in the transgenic AD mouse model overproducing Aβ1–42 (20).

Previous studies have shown that the prion protein binds with high affinity to relatively large (2–6 nm in diameter) toxic oligomers of Aβ1–42, but not to the monomeric form of the peptide (11, 19, 21). Such toxic oligomers are often observed as transient species during Aβ amyloidogenesis (25), although it is not clear whether they are on-pathway intermediates in the fibrillization reaction or rather off-pathway assemblies that are in rapid equilibrium with the monomer and become depleted during the course of the reaction. The present finding that when added to Aβ1–42 monomers, the prion protein not only prevents generation of these oligomers but also inhibits fibril formation at very low concentrations, corresponding to as little as 1:100 PrP:Aβ molar ratio. To put this in context, previously considered inhibitory effects have been proposed to be mediated by modulation of the p53 pathway (30, 31), molecular level mechanisms by which N1 interacts with potential binding sites (other than PrP C) on the membrane surface.

Although further studies are needed to fully characterize different types of Aβ-PrP complexes, the present findings have potentially important biological implications. In particular, it should be noted that the two domains of the prion protein (−95–110 sequence and the 23KRRPK27 N-terminal basic residues) required both for PrP interaction with preformed Aβ oligomers (11, 21) as well as for the inhibitory effect on Aβ assembly into toxic species are fully included in the N-terminal N1 fragment (residues 23–110/111), a polypeptide that is physiologically released from the membrane-anchored PrP C into extracellular space by α-cleavage with a disintegrin and metalloproteinase (ADAM) family metalloproteases (29). This α-secretase-derived N1 fragment of the prion protein was shown to protect cells from various proapoptotic challenges and play a neuroprotective role in vivo in a pressure-induced ischemia model of rat retina (30). Importantly, a recent study has shown that N1 can also protect primary cultured neurons against the toxic action of Aβ oligomers (31). Although these effects have been proposed to be mediated by modulation of the p53 pathway (30, 31), molecular level mechanisms by which N1 inhibits the toxicity of Aβ remain unknown. The present data provide a missing link in this regard, strongly suggesting that the primary event in this protective action could be direct interaction between N1 and early misfolded species of Aβ, resulting in the shift in Aβ aggregation pathway toward nontoxic assemblies.

Finally, it should be noted that the recombinant prion protein and its N-terminal fragments are able to alter the aggrega-

tion pathway of Aβ and inhibit fibril formation at very low concentrations, corresponding to as little as 1:100 PrP:Aβ1–42 molar ratio. To put this in context, previously considered inhibitors such as so-called β-sheet breaking peptides are typically effective only at much higher concentrations (32–34). Such a striking potency of the prion protein and its N-terminal fragments as inhibitors of the assembly of Aβ into toxic species may offer new avenues for pharmacological intervention in AD by using synthetic analogues/derivatives of these fragments.
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