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Structural and Functional Alterations in Amyloid- β Precursor Protein Induced by Amyloid- β Peptides

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Abstract

Alzheimer's disease-associated amyloid- β (A β) peptide is neurotoxic as an oligomer, but not as a monomer, by an unknown mechanism. We showed previously that A β interacts with the amyloid- β precursor protein (A β PP), leading to caspase cleavage and cell death induction. To characterize this structure and interaction further, we purified the extracellular domain of A β PP₆₉₅ (eA β PP) and its complex with A β oligomers (A β Os) of varying sizes, and then performed small angle X-ray scattering (SAXS). In the absence of any A β , eA β PP was a compact homodimer with a tight association between the E1 and E2 domains. Dimeric A β oligomers induced monomerization of eA β PP while larger oligomers also bound eA β PP but preserved the homodimer. Efficient binding of the larger oligomers correlated with the presence of prefibrillar oligomers, suggesting that the eA β PP binding is limited to a conformational subset of A β oligomers. Both forms of A β bound to eA β PP at the A β -cognate region and induced dissociation of the E1 and E2 domains. Our data provide the first structural evidence for A β -A β PP binding and suggest a mechanism for differential modulation of A β PP processing and cell death signaling by A β dimers versus conformationally-specific larger oligomers.

Keywords

Alzheimer's disease; amyloid- β oligomers; amyloid- β protein precursor; small angle X-ray scattering

INTRODUCTION

Alzheimer's disease (AD) affects more than five million people in the US, yet it is currently lacking any effective treatment. Although past interest has focused primarily on amyloid- β (A β) contained in plaques, previous research from our laboratory and others has shown that, at least in transgenic mouse models of AD, high levels of A β and plaque formation do not

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necessarily lead to the Alzheimer's phenotype [1]. Instead, both pre-plaque pathophysiology—possibly due to A β oligomers [2–4]—and distinct, plaque-related pathology appear to be involved.

A number of previous reports have demonstrated toxicity of A β based on chemical and physical effects on the cell, such as lysosomotropic detergent-like effects [5], metal binding [6], and generation of reactive oxygen species [7]. Recent, complementary results argue that signaling events, some of which are mediated by the amyloid- β precursor protein (A β PP) itself, also play a crucial role in the development of the AD phenotype [2, 8–12]. Furthermore, A β PP has recently been shown to be a receptor for netrin-1 [12], an axon guidance and survival factor; and to give rise to N-A β PP, a ligand for DR6 (death receptor 6) [13]. Thus, A β PP may mediate either trophic, anti-apoptotic events—when bound, for example, by netrin-1—or anti-trophic, pro-apoptotic events—when bound, for example, by A β [14–16].

Previous work has shown that A β interacts directly with A β PP within the A β -cognate region, leading to caspase cleavage of A β PP and cell death induction [14–15]. This may be a crucial event in AD pathogenesis, since, in the PDA β PP transgenic mouse model of AD, a mutation at the caspase cleavage site in A β PP prevents the synaptic loss, dentate gyral atrophy, memory loss, electrophysiological abnormalities, and neophobia associated with the AD phenotype, without reducing plaque formation or concentrations of soluble A β _{1–40} or A β _{1–42} [8, 17]. A β has been shown to bind readily to membranes [18–20] and to a variety of membrane proteins in addition to A β PP [21]. Therefore, in order to understand its binding to A β PP, it is important to use a simpler system to define the structural basis of this A β -A β PP interaction. These studies will help to identify modulators of this interaction, and may be useful in developing novel therapeutic agents for evaluation *in vivo*.

Understanding the A β -A β PP interaction can be broken down into three distinct parts, based on previous work. First, since A β forms oligomers of different sizes and degrees of toxicity [22–23], one of the key questions is whether A β PP binds to a specific type of oligomer or recognizes a broad variety of oligomers. Second, since A β binding effects A β PP signal transduction, it is also of interest as to whether A β binding causes conformational changes within the ectodomain of A β PP that could affect the binding of A β PP ligands such as netrin. Finally, dimerization of the ectodomain of A β PP has been shown to potentiate cell death [15, 24], therefore determining whether binding of A β oligomers changes the oligomerization state of the ectodomain is also important to understanding whether A β binding and dimerization of A β PP enhance cell death through the same pathways.

Understanding the A β -A β PP interaction on a molecular level is complicated by the size and flexibility of the A β PP ectodomain as well as the propensity of A β oligomers to aggregate at higher concentrations. Recent improvements in synchrotron data collection techniques allow characterization of the size, shape, and flexibility of molecular complexes in solution at low concentration [25] using small angle X-ray scattering (SAXS). In addition, for flexible molecules, SAXS data combined with the high-resolution crystal structures can reveal preferred domain orientation, domain interaction within multimers, and changes in flexibility [26]. Accordingly, in the current study, we utilized SAXS, cross-linking, and size

exclusion chromatography to study the extracellular domain of A β PP (eA β PP) with and without its structure bound to A β oligomers of varying size. We compared the effects of three different sizes of A β oligomers and found that A β binding affects both the conformation and the oligomerization state of the eA β PP multimers, and these effects are dependent on the size of the A β oligomers. Our data provide the first structural evidence for A β -A β PP binding, and suggest a mechanistic basis for observed functional effects of modulation of A β PP processing and neuronal cell death signaling by A β oligomers.

MATERIALS AND METHODS

Protein expression and purification

Fragments of the ectodomain of A β PP₆₉₅ were cloned into the pET-102/D-TOPO vector using a Champion Directional Cloning Kit (Invitrogen) to produce His-Patch thioredoxin fusion proteins. A stop codon was introduced after residue 624 to prevent transcription of the 6 $\times$ C-terminal His-Tag. The His-Patch thioredoxin fusion proteins containing either human A β PP residues 19–624 (eA β PP₁₉₋₆₂₄), 290–624 (eA β PP₂₉₀₋₆₂₄), or residues 575–624 (eA β PP₅₇₅₋₆₂₄) were purified by immobilized metal ion affinity chromatography (IMAC) using a HiTrap Chelating HP column charged with nickel sulfate and eluted with a 10 column volume gradient between 2 mM imidazole, 20 mM Bis-Tris pH 6.5, 100 mM NaCl, and 60 mM imidazole pH 7.4. eA β PP₁₉₋₆₂₄ or eA β PP₂₉₀₋₆₂₄ was then loaded onto a HiTrap Heparin FF column and eluted with a 10 column volume gradient between 20 mM Tris pH 7.0, 50 mM NaCl, and 20 mM Tris pH 7.0, 500 mM NaCl. eA β PP₅₇₅₋₆₂₄ was loaded onto a HiTrap Q FF column and eluted with a 10 column volume gradient between 20 mM Tris pH 7.4, 50 mM NaCl, and 20 mM Tris pH 7.4, 500 mM NaCl. After concentration, both proteins were additionally purified by size exclusion chromatography (HiPrep 26/60 Sephacryl S-200 column for eA β PP₅₇₅₋₆₂₄ and eA β PP₂₉₀₋₆₂₄ or an HiPrep 26/60 Sephacryl S-300 column for eA β PP₁₉₋₆₂₄) with a running buffer of 20 mM Bis-Tris pH 6.5, 100 mM NaCl, 2.6 mM EDTA, 0.002% azide. All HiTrap and HiPrep columns were obtained from GE-Healthcare. In addition to the three thioredoxin fusion proteins, eA β PP₂₃₀₋₆₂₄ was expressed as a fusion protein with maltose binding protein (MBP) using the pDEST-periHisMBP vector [27]. The MBP-eA β PP₂₃₀₋₆₂₄ fusion protein was purified using a similar protocol to that of the thioredoxin fusion proteins. The MBP was removed from the MBP-eA β PP₂₃₀₋₆₂₄ using a tobacco etch virus protease (TEV) site engineered into the protein. The TEV was purchased from Eton Bioscience. The MBP was separated from the eA β PP₂₃₀₋₆₂₄ using the Heparin FF column followed by size exclusion chromatography (HiPrep 16/60 Sephacryl S-300 column).

Preparation of A β peptides

A β ₁₋₄₀, A β ₁₋₄₂, A β ₁₋₂₈, A β ₁₂₋₂₈, A β ₁₇₋₄₂, A β ₄₂₋₁, and A β ₁₋₂₀ peptides were purchased from AnaSpec. The peptides were solubilized by dissolving 0.5 mg of peptide in 30 μ l of 100 mM NaOH, pH 11. The peptides were then diluted to 1 mg/ml using PBS (10 mM phosphate pH 7.4, 137 mM NaCl, 2.6 mM EDTA). 20-kDa and 70-kDa A β oligomers were produced by using size exclusion chromatography (SEC) to purify partially aggregated A β ₁₋₄₀ or A β ₁₋₄₂. The A β peptides were further diluted to 0.1 mg/ml with PBS and then incubated at 37°C for 0.5 h–3 days in a water bath. The 20-kDa and 70-kDa oligomers of

A β were then separated at 4°C using Superdex S-75 SEC (GE Healthcare) (20-kDa or 70-kDa, incubation time less than 7 h) or Superdex S-200 (70-kDa, incubation times greater than 7 h) using PBS as the running buffer. Column loads were adjusted so that the concentration of A β in the fractions was less than 50 μ g/ml.

Analysis of the partially aggregated A β ₁₋₄₀ and A β ₁₋₄₂ peptides with SEC (Superdex S-75) indicated that the dominant oligomer size was 26-kDa for A β ₁₋₄₀ and 20-kDa for A β ₁₋₄₂ after solubilization and dilution. Incubation at 37°C reduced the oligomer size of the A β ₁₋₄₀ to 20-kDa (Supplementary Fig. 1A; available online: <http://www.j-alz.com/issues/25/vol25-3.html#supplementarydata02>). The optimal incubation times had to be adjusted for each lot of A β and ranged from between 1–2 h for A β ₁₋₄₀ and 1.5–2.5 h for A β ₁₋₄₂. Consistent with previous observations [28], incubation of the A β ₁₋₄₂ produced a secondary peak eluting at 70-kDa at relative short incubation times (30 min to 2 h) (Supplementary Fig. 1B), while incubation for more than 6 h was required for A β ₁₋₄₀ to achieve a similar elution profile. Analysis of stored A β oligomers by SEC indicated that the location of peaks of the A β ₁₋₄₂ elution profile did not significantly change during storage at 4°C for up to 6 h (Supplementary Fig. 1B) or after one freeze-thaw cycle (not shown). Aliquots of all of the peptides were stored frozen at –20°C until use and then thawed at 4°C or were used immediately after purification.

The 7-kDa A β ₁₋₄₀ and A β ₁₋₄₂ peptides were produced by solubilizing the peptides following the method of Zhang et al., [29] and were then purified by SEC using the Superdex S-75 at 4°C with PBS as the running buffer. Since working with the A β peptides at 4°C and concentrations of less than 100 μ g/ml retards the formation of larger oligomers [28], this technique was used to extend the lifetime of the 7-kDa A β ₁₋₄₀ beyond 8 h. Due to the faster aggregation kinetics of A β ₁₋₄₂, the 7-kDa A β ₁₋₄₂ was difficult to produce in the quantities needed for the small angle X-ray scattering experiments. The 7-kDa A β ₁₋₄₂ was only used in the SEC experiments and was combined with the eA β PP fragments immediately after purification.

The A β ₁₋₄₀ and A β ₁₋₄₂ oligomers were characterized for A-11 reactivity using the rabbit polyclonal A-11 antibody (Invitrogen). One microgram A β oligomers, as determined using the Coomassie Plus Protein Assay (Pierce), were spotted onto nitrocellulose. The membranes were probed with 0.1 μ g/ml of A-11 in TBST with 5% milk. The secondary antibody used was ECL anti-rabbit horseradish peroxidase linked (GE Healthcare) at a 1:10,000 dilution. Complementary dot blots using the A β monoclonal antibodies 6E10 and 4 G8 at 0.05 μ g/ml and 1 μ g/ml, respectively, were used as controls.

Formation of complexes between A β peptides and eA β PP fragments

To form these complexes, A β peptides (0.05–0.1 mg/ml) and eA β PP fragments (0.05–0.1 mg/ml) were incubated for 1–3 h on ice. To ensure that the size-profile of the oligomers was close to the SEC results, the oligomers were stored on ice for less than 30 min before being added to the eA β PP fragments. The A β -eA β PP fragment mixtures were then concentrated using Amicon Ultra concentrators with a 5-kDa cutoff and purified by SEC (Superdex S-200, GE Healthcare) with a running buffer of 10 mM Tris pH 7.4, 50–125 mM sodium chloride, 2.6 mM EDTA, and 0.002% (w/v) sodium azide. One to two 0.5-ml fractions were

pooled from the center of the relevant peak and concentrated at 4°C for analysis. Purity was assessed by SDS-PAGE. All samples were stored at 4°C for 6–12 h before SAXS analysis.

X-ray scattering data collection

Small-angle X-ray scattering (SAXS) data were collected using protein concentrations in the range of 0.1–3 mg/ml and an X-ray wavelength of 1.11Å at beam line 12.3.1 (Advanced Light Source). Samples of the running buffer from the size exclusion columns were used for buffer subtraction. Data were integrated with software customized for the beam line and processed with the program PRIMUS [30]. The program GNOM [31] was used to calculate the maximum dimension and the radius of gyration and to estimate the intensity of the scattering at zero angle for higher concentration samples. The dimensional data for each sample are summarized in Table 1. Although dilutions of each sample were analyzed to concentrations of approximately 0.2 mg/ml, no significant differences were observed in the dimensional data across the concentration ranges shown in Table 1.

Cross-linking studies

Aliquots of eAβPP₁₉₋₆₂₄ in PBS buffer were incubated with either SEC-purified Aβ₁₋₄₀ or Aβ₁₋₄₂ oligomers at a 1:20 molar ratio for 30 min at room temperature. 25 mM bis(sulfosuccinimidyl)suberate (BS3) was added to a final concentration of 2.5 mM. The final concentration for eAβPP₁₉₋₆₂₄ was 10 μM. The aliquots were further incubated for 40 min at room temperature, before the reaction was stopped by the addition of 1 M Tris pH 7.4 sufficient for a final concentration of 20 mM Tris pH 7.4. The samples were frozen at –20°C prior to analysis with reducing SDS-PAGE. The reactivity to different antibodies was assessed by Western blotting. The primary antibody concentrations were 1 μg/ml for 11 H3 (Santa Cruz Biotechnology) and 4 G8 (Millipore) and 0.5 μg/ml for 6E10 (Covance). R1736 (gift from Dr. Dennis Selkoe, Harvard University) was used at a 1:1000 dilution. The bands were integrated using ImageJ [32].

RESULTS AND DISCUSSION

In order to evaluate the Aβ–AβPP interaction, our experiments addressed three questions: a) whether AβPP binds to a specific type of Aβ oligomer or recognizes a broad variety of oligomers; b) whether binding of Aβ oligomers changes the oligomerization state of the AβPP ectodomain; and c) whether Aβ binding causes conformational changes within the ectodomain of AβPP. To provide the tools to address these questions, we generated batches of Aβ oligomers that varied in size and conformation, as well as a series of truncation fragments of the ectodomain of AβPP (eAβPP). SEC, cross-linking, and a conformation-specific antibody were used to characterize the Aβ oligomers. SEC and small-angle x-ray scattering were similarly used to characterize the eAβPP fragments. The complexes of different Aβ oligomers with the eAβPP fragments were then studied using similar methods to determine the effects of Aβ binding on the conformation and oligomerization state of the ectodomain of AβPP.

Characterization of A β Oligomers

Recently, there has been an emerging consensus that small, soluble A β oligomers, rather than insoluble A β fibrils, represent the predominant toxic species in increasing apoptosis, decreasing neuronal synaptic plasticity and impairing memory [23]. Different types of soluble A β oligomers have been described from synthetic A β peptides, cell culture systems, and transgenic mouse or human brain samples, such as protofibrils, A β -derived diffusible ligands (ADDLS), globulomers, low-n oligomers, and A β *56 [22]. Since our goal was to determine whether A β PP binds to A β oligomers in general, we purified low molecular weight A β oligomers in three different size ranges: 7-kDa, approximately 20 kDa, and approximately 70-kDa. These size ranges were obtained by optimizing the aggregation kinetics of A β to produce oligomers of reproducible size that could be purified by SEC. The smaller oligomers were produced by incubating 9 μ M A β ₁₋₄₀ or A β ₁₋₄₂ in PBS at 37°C for 0.5–3 h prior to purification by SEC on a calibrated Superdex 75 column. Under these conditions, A β ₁₋₄₀ or A β ₁₋₄₂ produced oligomers in three sizes (Fig. 1A). The 7-kDa A β ₁₋₄₀ was a mixture of monomers and dimers, as evidenced by BS3 cross-linking (Fig. 1B). Cross-linking the 20-kDa A β ₁₋₄₂ generated trimers but not dimers, suggesting that the larger oligomers are composed of trimeric subunits. Treatment with BS3 modifies lysines near the 6E10 epitope (residues 597–613), as evidenced by the absence of the monomer band in the 7-kDa BS3-treated A β ₁₋₄₀ and the trimer band in the 20-kDa BS3-treated A β ₁₋₄₂. This differential recognition after BS3 treatment indicates that the trimers and dimers in the two cross-linked samples are derived from oligomers with different conformations. A β ₁₋₄₀ and A β ₁₋₄₂ low molecular weight oligomers produced by similar solubilization methods have been previously characterized using cross-linking and FRET. These studies identified the three sizes of SDS-labile A β oligomers produced by solubilizing with sodium hydroxide and then diluting into PBS at pH 7.4 as dimers (7-kDa peak) [33], a mixture of pentamers and hexamers (20-kDa peak), and dodecamers to octadecamers (70-kDa peak) [34]. A third study, using mass spectrometry to evaluate A β solubilized with sodium hydroxide and then diluted into ammonium acetate at pH 7.4, concluded that A β ₁₋₄₂ forms primarily dimers, tetramers and dodecamers [35].

Previous work on the fibrillization pathways of A β demonstrated that A β oligomers of similar sizes adopt distinct conformations dependent on the aggregation conditions, and these conformations can be distinguished both immunologically and by their relative toxicities [36]. Because A β PP has been shown to co-precipitate from rat cortical neurons with A β fibrils produced at 37°C, but not with amorphous A β aggregates produced by incubation of A β at 24°C [21], whether eA β PP showed some form of specificity toward pre-fibrillar forms of A β was of particular interest. By comparing the effects of these two types of preparations, we could determine whether eA β PP₁₉₋₆₂₄ has a higher affinity for oligomers that contain the epitope characteristic of pre-fibrillar A β oligomers.

Characterization of the larger oligomers (70-kDa and 20-kDa) prepared using the techniques described above revealed that the 70-kDa A β ₁₋₄₀ pool did not react with the conformation-specific antibody A-11. This antibody recognizes an epitope that is displayed by soluble pre-fibrillar oligomers [3]. To generate a pool of A β ₁₋₄₀ enriched in pre-fibrillar oligomers, A β ₁₋₄₀ oligomers were allowed to aggregate at 37°C for three days before purification by

SEC. This pre-aggregation step resulted in a dominant A β ₁₋₄₀ species that eluted from the calibrated Superdex 200 column at 70-kDa (Fig. 1C). The purified A β ₁₋₄₀ oligomers generated two cross-linked species close to 60-kDa in relative molecular mass (Fig. 1D), suggesting that this species is composed of lauryldodecylsulfate (LDS)-soluble globular oligomers formed from 12–18 A β ₁₋₄₀ molecules. Similar populations of 70-kDa A β ₁₋₄₂ oligomers were generated by incubation at 37°C for 6 h. Dot blots probed with the A-11 antibody confirmed the presence of pre-fibrillar oligomers in both samples. Similar differences in kinetics have been previously observed between A β ₁₋₄₀ and A β ₁₋₄₂ [3].

Homodimerization of the A β PP ectodomain occurs through the A β -cognate region

Since a ligand-receptor type interaction should require only the ectodomain of A β PP, we engineered four constructs containing fragments of the ectodomain and expressed them in *E. coli* (Fig. 2). By comparing the results from a series of constructs, the A β -binding site could be localized and the effect of A β binding on the conformation of eA β PP could be determined. The largest construct, which was the primary construct used for these studies, contained the entire ectodomain (residues 19–624 (eA β PP₁₉₋₆₂₄). In the second construct (eA β PP₂₉₀₋₆₂₄), the two amino-terminal domains previously implicated in A β PP dimerization [37, 38] were deleted from the ectodomain. Although each domain is a true structural domain, these two domains are collectively referred to as the E1 domain. The residues between 290 and 575 are similarly referred to as the E2 domain [39]. In the third construct (eA β PP₅₇₅₋₆₂₄), only the region C-terminal to the last folded domain was retained. All three constructs contain an N-terminal His-Patch thioredoxin fusion partner. The fourth construct encoded a protein (eA β PP₂₃₀₋₆₂₄) derived from a maltose binding protein (MBP) fusion. For this protein, the MBP was removed by cleavage at a tobacco etch virus (TEV) protein site to generate eA β PP₂₃₀₋₆₂₄. The carboxyl-terminus of each of the four proteins is the A β ₁₋₂₈ sequence (A β -cognate region), which includes the α -secretase and β -secretase cleavage sites. All four were immunoreactive with the 6E10 antibody (whose epitope is at residues 597–613) and the 4 G8 antibody (whose epitope is at residues 612–621), indicating that the A β ₁₋₂₈ cognate region was fully expressed.

Small angle x-ray scattering (SAXS) analysis of the purified proteins (Table 1) showed that, at pH 7.4, eA β PP₁₉₋₆₂₄ was significantly larger than sA β PP (eA β PP₁₉₋₆₁₂), which has been shown previously to be a monomer [40]. Relative molecular weight estimations, based on comparison of the scattering by eA β PP fragments to the scattering of reference proteins with known molecular masses, indicated that all of the eA β PP fragments were homodimeric at concentrations below 1 mg/ml. Consistent with the SAXS analysis, at concentrations between 10 μ g/ml and 1 mg/ml, these fragments eluted from a calibrated SEC at times corresponding to estimated molecular masses approximately twice their molecular weights: 210-kDa for eA β PP₁₉₋₆₂₄, 110-kDa for eA β PP₂₃₀₋₆₂₄, 100-kDa for eA β PP₂₉₀₋₆₂₄, and 36-kDa for eA β PP₅₇₅₋₆₂₅.

Shape reconstruction techniques (as implemented in BUNCH [38]) applied to the SAXS data indicated that eA β PP₁₉₋₆₂₄ forms a parallel homodimer (Fig. 3). The computer program BUNCH uses molecular dynamics, biochemical constraints, and the crystal structures of the individual domains to find a model that best fits the SAXS data. The agreement between the

model and the SAXS data is measured by the X -value. X -values below 2.5 indicate a very good fit for protein SAXS data while x -values above 5.0 indicate that the BUNCH calculations failed to converge and the resulting model is not related to the data. To model the eA β PP₁₉₋₆₂₄ dimer, we derived sets of constraints based on other studies. For example, one study concluded that fragments of A β PP containing residues 19–305 (the E1 domain) multimerize by a redox-dependent mechanism [41]. Similarly, association of the N-terminal domains in the context of full-length A β PP expressed in cells has been shown to promote homodimerization and therefore could potentially affect A β PP-mediated signaling [42]. Both of these studies suggest that the N-terminal domains might be involved in the dimer interface. However, limitation of the dimerization interface to the C-terminal tail of the ectodomain of A β PP is consistent with previous structural studies that concluded that sA β PP which contains the N-terminal domains, is a monomer [43] that requires the presence of heparin to interact with the full-length ectodomain of A β PP to dimerize [24]. To clarify these issues, models of both anti-parallel and parallel dimers, in which either the N-terminal growth factor-like domain (GFLD), the Cu-binding domain, the thioredoxin fusion moiety, or the A β -cognate region was constrained to be in the dimer interface, were tested against the SAXS data. All of these calculations produced models inconsistent with the SAXS data ($x > 4.5$), except for the model in which the A β -cognate region contributed to the dimer interface ($x = 1.5$, $(0.013 < q < 0.3)$ (Fig. 3). This model is a compact parallel homodimer with two interfaces: the major dimer interface is composed of residues 575–624 in the C-terminal tail of eA β PP, and the minor dimer interface is located within the RERMS domain. In this model, the E1 domain (GFLD and Cu-binding domains together) packs tightly against the E2 (RERMS-CAPPD) domain to form a compact monomeric unit. The orientation of the E1 and E2 domains is highly similar to that found in the SAXS model of sA β PP α [43]. Neither of the thioredoxin domains contact the eA β PP molecules, but each instead appears as an individual domain tethered by the linker to a much larger molecule.

The smaller dimerization interface within the RERMS domain corresponds to a helix-loop-helix structure (residues 445–495). This same region was shown to participate in a homodimeric interface in the RERMS-CAPPD (central A β PP domain) crystal structure (PDB code 1RW6 [44]). Thus, this region of eA β PP has a propensity to stabilize self-association. As in the crystal structure, the RERMS-CAPPD units are oriented anti-parallel in the BUNCH model even though the overall orientation of the two eA β PP₁₉₋₆₂₅ molecules is parallel. Models that inverted the orientation of the RERMS-CAPPD unit by stretching the acidic region had a poor fit to the SAXS data ($X > 3.5$), indicating that it is indeed the RERMS domain that contributes to the interface. The RERMS domain may not be the only contributor to the interface, however, since some models with a good fit to the SAXS data ($1.6 < X < 2.0$, $(0.013 < q < 0.3)$) placed residues of the flexible acidic region within the margin of this interface. This uncertainty results from the fact that BUNCH models unstructured regions, such as the acidic region, as a flexible chain of amino acid-type balls. At the resolution of the SAXS data (20Å), small variations in the path of the chain do not have as large an impact on the fit as a rotation of one the domains.

Similarly, the SAXS model is too low in resolution to determine which residues in the 575–624 region participate in the major dimer interface. The folding of the C-terminal tails is a

representation of the volume likely to be occupied by those residues. Therefore, unlike the CAPPD-RERMS domain for which the crystal structure is known, the model cannot be used to locate specific features within the 575–624 region which might participate in the dimer interface. However, the participation of the A β -cognate region within the major dimerization interface is consistent with the competition experiments presented below, since A β fragments containing the corresponding residues (A β ₁₋₂₈) were sufficient to disrupt the eA β PP₁₉₋₆₂₄ dimer. Similarly, participation of residues 613–624 in self-association is consistent with the lack of self-association of sA β PP residues (19–612) [40]. Participation of residues 613–624 is also supported by a nuclear magnetic resonance study of the C-terminal 99 amino acid (C99) fragment of A β PP (A β PP₅₉₆₋₆₉₅) in a detergent bilayer demonstrated that the amides of residues in the A β -cognate region had deuterium exchange rates typical of residues buried within a protein-protein interface [45]. Other studies have also suggested that the A β -cognate region plays a critical role in the formation of A β PP dimers, based on mutations and domain swapping with receptors related to A β PP [42].

Evaluation of A β oligomer binding to the A β PP₆₉₅ ectodomain

To confirm that A β ₁₋₄₀ peptides bind directly to the ectodomain of A β PP₆₉₅ (eA β PP), the eA β PP fragments were incubated with different-sized A β oligomers at 4°C for 1 h under saturating conditions (a molar ratio of 1:20 eA β PP to A β molecules). Keeping the A β concentration below 10 μ M [18], the temperature near 4°C, and the pH > 7.0 [28] disfavors A β fibril formation and allows the A β oligomers to interact with the eA β PP fragments at concentrations that have been shown to be effective for inducing cell death [46]. Because the pools of A β oligomers purified by size are likely to be conformationally heterogeneous, saturating conditions as judged by the presence of a large excess A β peak were used in all incubations, thus allowing the interacting species to bind eA β PP. SEC was then used to separate the ectodomain fragments from the excess A β peptides, and then the fractions containing the ectodomain fragments were analyzed by SDS-PAGE and Western blotting. For the incubation containing the A-11 positive A β ₁₋₄₀ oligomers and eA β PP₁₉₋₆₂₄, this analysis verified that A β ₁₋₄₀ was included in the peak eluting at 260-kDa (Fig. 4A). The size of this shift was consistent with a large globular A β ₁₋₄₀ oligomer binding to a dimer of eA β PP₁₉₋₆₂₄. The excess A β ₁₋₄₀ eluted at the expected elution volume for A β dimers. The breakdown of the excess 70-kDa A β ₁₋₄₀ into smaller units suggested that the optimal size for A β oligomers to bind to eA β PP₁₉₋₆₂₄ is less than 70-kDa. Similar results were obtained for the incubations containing 7-kDa A β ₁₋₄₀ and eA β PP₁₉₋₆₂₄ or eA β PP₅₇₅₋₆₂₄ (Fig. 4B). 7-kDa A β ₁₋₄₂ prepared under similar conditions also co-eluted with eA β PP₅₇₅₋₆₂₄ (not shown). These observations confirmed our previous results in cell-based assays, which indicated that at least one binding site is contained within residues 575–624 [15]. They also confirm that A β oligomers of different sizes bind to eA β PP raising the question as to whether a specific type of A β conformation is required for binding.

Differential binding of A-11-positive and A-11-negative 70-kDa A β oligomeric species to eA β PP

To determine whether the interaction between the eA β PP and the A β species showed specificity, we incubated eA β PP fragments with purified A β oligomers in all three size ranges, but prepared using different methods. As shown in Table 1, species of A β in all three

size ranges co-eluted with the fragments of the ectodomain of eA β PP. However, the effect of oligomer binding on the apparent size of the eA β PP fragments as measured by SEC and SAXS was dependent on the size of the oligomers, the method used to produce the oligomers, and whether or not the oligomers were reactive to the A-11 pre-fibrillar specific antibody.

The effectiveness of the 70-kDa oligomers did indeed vary by the method of production and their reactivity to A-11 (Fig. 5A). The A-11-positive 70-kDa oligomers, like those in Fig. 4, significantly increased the apparent molecular weight of the eA β PP₁₉₋₆₂₄ dimer (as determined by SEC) independent of whether the oligomers contained A β ₁₋₄₀ or A β ₁₋₄₂. However, A-11-negative batches of 70-kDa A β ₁₋₄₀ oligomers (0.5–3 h of incubation at 37°C) had no significant effect on the size of the eA β PP₁₉₋₆₂₄. Qualitatively, the A β band from the complexes was either much more difficult to detect (requiring two orders of magnitude more sensitivity (Pierce Supersignal West Femto Kit versus the Pierce ECL Plus kit) or not detected in the Western blot analysis of the eA β PP₁₉₋₆₂₄ fraction, indicating that less A β was bound to the eA β PP fragment after incubation with the A-11-negative oligomers than with the A-11-positive oligomers. The greater efficacy of the A-11-positive oligomers to bind suggests that binding may correlate with the presence of a structure found in pre-fibrillar oligomers.

A differential efficacy of binding was also observed between 20-kDa A β produced at 24°C and 37°C. Like the 70-kDa oligomers, the 20-kDa A β ₁₋₄₂ oligomers produced at 37°C were A-11-positive. Although our experiments do not address the secondary structure of the oligomers, incubation of micromolar solutions of A β ₁₋₄₀ or A β ₁₋₄₂ at 37°C has been shown to increase the amount of β -sheet secondary structure present, without substantially changing the size of the oligomers [47]. Soluble oligomeric A β in the 20-kDa size range produced by incubation at 37°C under similar conditions to those used in this study has been shown by NMR studies to have a higher proportion of β -pleated sheet structures (which can be parallel β -sheet or anti-parallel β -sheet) than oligomers produced at 24°C [48–49]. The 20-kDa A β oligomers formed at 37°C are also thought to be the building blocks of the larger pre-fibrillar oligomers, which is consistent with the A-11 reactivity of the A β ₁₋₄₂ oligomers [36].

Although it is not possible to conclude from these experiments that eA β PP interacts specifically with prefibrillar oligomers, our experiments suggest that there is a preferential binding for oligomers that are present under conditions that promote the formation of prefibrillar oligomers. A key point is that not all oligomers bind to eA β PP. Generation of batches of oligomers that were inefficient at co-eluting with the eA β PP fragments indicates that our results are not due to nonspecific interactions between the protein and “sticky” oligomers, but rather reflect binding of a select group of A β conformations to eA β PP.

Binding of 20-kDa A β oligomers reproducibly increases eA β PP₁₉₋₆₂₄ dimerization

To determine whether A β oligomer binding affects the oligomerization state of eA β PP, the complex of the 20-kDa A β oligomers and eA β PP were studied with SAXS analysis. The 20-kDa A β oligomers were used because they are more readily produced in the quantities required for SAXS than the 70-kDa A β oligomers. Incubation with A-11-positive 20-kDa

oligomers reproducibly produced a larger size complex ($n = 4$) (Table 1, Fig. 5A). Production of the complex was not dependant on purification of the 20-kDa oligomers. As shown in Supplementary Fig. 2, A β ₁₋₄₂ incubated for 2 h at 37°C to generate an A-11-positive sample and then immediately mixed with eA β PP₁₉₋₆₂₄ gives identical results to the addition of purified 20-kDa A β ₁₋₄₂ to eA β PP₁₉₋₆₂₄. In both cases, the excess A β ₁₋₄₂ elutes from the column as a mixture of 20-kDa and 7-kDa A β ₁₋₄₂. This suggests the 20-kDa A β ₁₋₄₂ is not simply associating into 70-kDa oligomers during the co-incubation and then binding to the eA β PP₁₉₋₆₂₄. The increase in size (15–20%) of the eA β PP₁₉₋₆₂₄ dimer as measured by SAXS corresponds to 24-to 32-kDa, which is within the experimental error of the expected increase in mass of binding due to the binding of six to eight A β molecules to the eA β PP₁₉₋₆₂₄ dimer. Denaturing SDS-PAGE on the 20-kDa A β ₁₋₄₂ eA β PP₁₉₋₆₂₄ complex detected the presence of monomeric and LDS-resistant trimers of A β ₁₋₄₂, but no larger species (Supplementary Fig. 2D). Therefore, this increase in mass is consistent with the binding of oligomers within the envelope of the 20-kDa SEC peak. The linearity of the Guinier plots indicates that these larger A β -eA β PP₁₉₋₆₂₄ dimers are a homogeneous species that are stable in solution at 4°C for the 10–12 h required for the experiment (Fig. 5B). This homogeneity suggests that the eA β PP fragments are selecting oligomers of similar size and shape from the 20-kDa oligomer pool.

Binding of 7-kDa (dimeric) A β decreases eA β PP dimerization

In contrast to the larger oligomers produced at 37°C, the 7-kDa A β decreased the apparent size of the eA β PP₁₉₋₆₂₄ (Fig. 5A). This effect was highly reproducible for the 7-kDa A β ($n = 5$). The efficacy of the 7-kDa A β ₁₋₄₀ or A β ₁₋₄₂ binding was independent of the temperature at which it was generated. SAXS analysis of these complexes estimated their molecular weight as close to that of monomeric eA β PP₁₉₋₆₂₄, indicating that the decrease in size was due to shifting the monomer-dimer equilibrium of eA β PP₁₉₋₆₂₄ in favor of the monomer (Table 1 and Fig. 5B). Incubation of the 7-kDa A β also decreased the size of eA β PP₂₉₀₋₆₂₄ and eA β PP₅₇₅₋₆₂₄, consistent with a binding site within the residues 575–624, which is common to all of the fragments (Table 1). Since the cross-linking of the 7-kDa A β indicated a predominance of dimers, we asked whether dimerization of A β was required to shift the eA β PP₁₉₋₆₂₄ monomer-dimer equilibrium. To answer this question, we analyzed the complex of the A β PP ectodomain fragments with partial A β peptides that do not aggregate at 4°C. As assessed by SEC, the size of eA β PP₁₉₋₆₂₄ decreased to that expected of a monomer after incubation at pH 7.4 with A β ₁₂₋₂₈, A β ₁₋₂₀, and A β ₁₋₂₈, 7-kDa A β ₁₋₄₀ or 7-kDa A β ₁₋₄₂. A β ₁₇₋₄₀ was less effective at shifting the monomer-dimer equilibrium of eA β PP₁₉₋₆₂₄ in favor of the monomer, and reverse peptide A β ₄₂₋₁ had no effect (Fig. 5C). This pattern suggests that residues 12–20 within monomeric A β are particularly potent at shifting the monomer-dimer equilibrium of eA β PP₁₉₋₆₂₄ in favor of the monomeric eA β PP₁₉₋₆₂₄ under saturating conditions. A β ₁₂₋₂₈ and A β ₁₋₂₀ are random coil monomers in solution, while A β ₁₋₂₈ is a monomer with significant helical content [50]. Therefore, our results suggest that binding of monomeric or dimeric, but not higher order oligomeric, A β fragments to the ectodomain of A β PP disrupts the eA β PP₁₉₋₆₂₄ dimers.

Summary of results of A β oligomer binding to the cognate region of eA β PP

The opposing effects of the 7-kDa A β oligomers and the 20-kDa A β oligomers may be due to the different number of A β sequences exposed on the surface of the oligomers. This hypothesis would account for the similar binding efficacy of 70-kDa A β and 20-kDa A β to eA β PP and their association with the fibrillization pathway. Pre-fibrillar oligomers are thought to result from the stacking of β -sheets formed by a conformational change of the A β peptide [22]. For at least one type of similarly-sized oligomer, this stacking of β -sheets has been shown to result in a curved β -sheet structure with an exposed A β sequence at each end [49]. Such soluble oligomers could bind two eA β PP molecules at once by recognition of the A β -cognate region thus creating a stable eA β PP homodimer- A β oligomer complex. Since the oligomer size is determined by the number of peptides buried within the stack, this type of oligomer would present two exposed A β sequences independent of its size. This reasoning makes the prediction that, although all of these three sizes of A β oligomer interact with the A β [notdef]cognate region, the footprint of the 20-kDa and 70-kDa oligomers should be similar, while the footprint of the 7-kDa oligomers should be distinctly different.

Larger A β oligomers protect K-612 from chemical modification

To test this prediction, we conducted a footprinting experiment on the complexes with the cross-linking agent BS3. Reaction of eA β PP₁₉₋₆₂₄ with a large excess of BS3 should modify most of the exposed lysine residues, whereas those buried within the protein-protein interface should remain unmodified. Preliminary experiments using a 250-fold excess of BS3 demonstrated that exposure to the BS3 dramatically decreased the recognition of eA β PP₁₉₋₆₂₄ by the 6E10 antibody specific to the A β cognate region, although both monomer and dimer bands of eA β PP₁₉₋₆₂₄ could be detected with Sypro Ruby staining (Fig. 6A). Another example of this effect is shown in Fig. 1B, where exposure to BS3 eliminated detection of the monomer band by 6E10 for the 7-kDa A β ₁₋₄₀, but not for the 20-kDa A β ₁₋₄₂. Reaction with BS3 most likely reduced the binding affinity of this antibody by modifying the lysine (K-612) within its epitope (Fig. 6B). Incubation of eA β PP₁₉₋₆₂₄ with a five-fold molar excess of A-11- positive 70-kDa A β ₁₋₄₀ partially restored the immunoreactivity, but incubation with even a twenty-fold excess of 7-kDa A β ₁₋₄₀ did not. This result is consistent with a significant protection of K-612 when eA β PP₁₉₋₆₂₄ binds to A-11-positive 70-kDa A β ₁₋₄₀, but not to 7-kDa A β ₁₋₄₀. Because the antibody recognizes both eA β PP₁₉₋₆₂₄ and A β , we cannot resolve whether the protected lysine is on the protein, the peptide or both. However, the difference in immunoreactivity indicates that the conformations of the complexes between 7-kDa A β ₁₋₄₀ and A-11-positive 70-kDa A β ₁₋₄₀ with eA β PP₁₉₋₆₂₄ are indeed different.

This conformational difference was explored by expanding the panel of antibodies to more fully probe the A β -cognate region of eA β PP. When the Western blot conditions were adjusted so that the BS3-treated monomeric eA β PP₁₉₋₆₂₄ could be detected, no significant difference was observed for a mixture of monoclonal antibodies (5A3/1 G7) specific for the E2 domain (residues 290–574) upon BS3 treatment for eA β PP₁₉₋₆₂₄ alone or incubated with either 7-kDa A β ₁₋₄₀ or 20-kDa A β ₁₋₄₂. In contrast, there was a systematic increase in immunoreactivity for antibodies to the A β -cognate region upon addition of either 7-kDa A β ₁₋₄₀ or 20-kDa A β ₁₋₄₂, indicating protection of the lysines within the A β cognate region

(Fig. 6C). As in the experiment shown in Fig. 6A, incubation with 7-kDa A β ₁₋₄₀ was the exception in that it did not significantly increase 6E10 immunoreactivity. Similarly, an antibody with the adjacent epitope, R1736, also showed a large difference in immunoreactivity between incubation with 7-kDa A β ₁₋₄₀ and 20-kDa A β ₁₋₄₂. This increase in immunoreactivity is most likely due to protection of the lysines on the protein because of the following three observations. First, the only species detected by 6E10 or 4 G8 in the control experiments (i.e., A β without eA β PP₁₉₋₆₂₄) were monomers and trimers as shown in Fig. 1B. Second, an antibody specific for the A β whose epitope contains the N-terminal amino-group of A β (11 H3) did not detect the bands assigned to monomeric eA β PP₁₉₋₆₂₄ independent of BS3-treatment. Finally, the R1736 antibody that does not recognize A β showed the same pattern of differential detection as 6E10 and 4 G8. Therefore, it is likely that the large increase in immunoreactivity was due to protection of K-612 on the protein. Enhanced protection of K-612 by the larger oligomers is also consistent with previous observations that soluble oligomers of A β can decrease α -cleavage of A β PP *in vivo* (for review [22]) because the α -cleavage site is between K-612 and L-613. Finally, protection of K-612 by both the A-11 positive A β ₁₋₄₀ and 20-kDa A β ₁₋₄₂ indicates that, in agreement with the conclusions of the SAXS and SEC analyses, the complex of eA β PP₁₉₋₆₂₄ with 20-kDa A β is more similar to the complex with A-11 positive 70-kDa A β ₁₋₄₀ than to the complex with 7-kDa A β ₁₋₄₀.

A β oligomer binding to eA β PP₁₉₋₆₂₄ increases conformational flexibility of eA β PP₁₉₋₆₂₄

The results presented above show that, although both dimeric A β and oligomeric A β (20-kDa and 70-kDa) bind eA β PP homodimers, the former split the homodimers into monomeric eA β PP in a heteromeric complex with A β , whereas the latter stabilize the eA β PP homodimer. This raises the question as to whether the binding of A β oligomers to eA β PP induces alterations in the secondary or tertiary structure of eA β PP. To address this question, SAXS analysis was used to compare the conformational flexibility of the eA β PP dimer in the presence and absence of A β .

As illustrated in Fig. 7A, the Kratky plot has a characteristic shape depending on whether the molecule is globular (i.e., spherical or elliptical), rod-shaped or mixed folded domains and random coil [51]. For globular proteins, the Kratky plot has an inverted parabolic shape at low scattering angles and then approaches zero at large scattering angles. For molecules that are highly flexible or contain only random coil, the Kratky plot is a straight line whose slope is proportional to the molecular weight. For molecules that have both globular domains and random coil regions, the Kratky plot is a composite of the two types, and typically has some curvature at low scattering angles and then transitions to a straight line at large scattering angles. Compact rod-like proteins can be distinguished from highly flexible proteins because the Kratky plot for a compact rod plateaus at large scattering angles.

In protein fragments such as the eA β PP fragments, which contain both compact domains and regions predicted to be random coil, changes in the shape of the Kratky plot are a qualitative indicator of the relative flexibility of the random coil regions. For the eA β PP₁₉₋₆₂₄ dimer (Fig. 7B), the Kratky plot displays the shape characteristic of a compact rod-like protein with globular domains, which, as discussed below, is supported by

the model generated independently from shape reconstruction techniques. This curve indicates that the dimer adopts a compact form in which there is relatively little flexibility. Incubation of eA β PP₁₉₋₆₂₄ with either the 7-kDa A β ₁₋₄₀ or 20-kDa A β ₁₋₄₂ increases the flexibility of the molecule. Similar results were obtained for all of the complexes listed in Table 1. The larger slope of the Kratky plot derived from 20-kDa A β ₁₋₄₂ incubation than from 7-kDa A β ₁₋₄₀ is consistent with the greater molecular weight of the 20-kDa A β ₁₋₄₂ complex.

In agreement with the Kratky plots, the monomeric complex of eA β PP₁₉₋₆₂₄ was best fit as an ensemble of flexible molecules, utilizing the program EOM [52] ($\chi = 1.5$, $(0.013 < q < 0.3)$). The added mass of one A β ₁₋₄₀ molecule was accounted for by adding 40 additional residues to the C-terminal tail. In these models, the acidic region and residues 575–624 behave as random coils in contrast to the compact conformation modeled in the eA β PP₁₉₋₆₂₄ dimer. Within the ensemble, the two regions vary greatly in length. For example, the closest distance between the E1 and E2 domains varied from 3.5–50 Å, with a mean of 14 Å. For comparison, the closest distance between E1 and E2 is 3.5 Å within the monomeric unit of the eA β PP₁₉₋₆₂₄ dimer.

To determine whether the increase in flexibility was due to the C-terminal unstructured region (residues 575–624) or the acidic region, we also examined the Kratky plots of eA β PP₂₉₀₋₆₂₄, which lacks the E1 domain and the acidic region (Fig. 7C). The Kratky plot of the eA β PP₂₉₀₋₆₂₄ dimer is typical of a compact rod, consistent with the rod-like shape of the E2 domain [44]. As for eA β PP₁₉₋₆₂₄, incubation of eA β PP₂₉₀₋₆₂₄ with the 7-kDa A β ₁₋₄₀ produced a large increase in flexibility, presumably due to the release of constraints on the C-terminal residues (575–624) upon formation of the monomer. Surprisingly, very little change was seen in the shape of the Kratky plot for the incubation with 20-kDa A β ₁₋₄₂. A similar result was obtained for 20-kDa A β ₁₋₄₀. Although both Western blotting and the increase in size of the complex verified the presence of A β ₁₋₄₂ in the samples, the shape and relative flexibility of the dimeric eA β PP₂₉₀₋₆₂₄ 20-kDa-A β ₁₋₄₂ complex was very similar to that of the eA β PP₂₉₀₋₆₂₄ dimer. This result is consistent with a model in which the larger A β oligomers bind to the existing eA β PP dimer through the C-terminal residues. It also suggests that the large increase in flexibility observed for the analogous eA β PP₁₉₋₆₂₄ complex requires the presence of the E1 domain and the acidic region. Since expansion of the acidic region is sufficient to account for the change in size of the complex, the expansion of the 20-kDa A β oligomer-eA β PP₁₉₋₆₂₄ complex is most likely due to dissociation of the E1 domain from the E2 domain analogous to what occurs with the monomeric 7-kDa-A β -eA β PP₁₉₋₆₂₄ complex.

Although this analysis does not address a mechanism for the dissociation of the E1 domain, it has been shown previously that an isolated fragment of the E1 domain binds A β fibrils [53]. Therefore, the expansion could result from one or a combination of two potential mechanisms: conformational change within the eA β PP₁₉₋₆₂₄ dimer due to A β -binding to the A β [notdef]cognate region, or A β oligomers binding outside the A β -cognate region and competing with the binding of E1 to E2. Figure 8 illustrates the proposed differences in flexibility between the 7-kDa A β complex, the 20-kDa A β complex, and the eA β PP₁₉₋₆₂₄ dimer. In summary, our results indicate that eA β PP₁₉₋₆₂₄ adopts three distinct

conformations: 1) the native homodimer; 2) a flexible monomer formed by interaction with dimeric A β ; and 3) a flexible dimer formed by binding larger A β oligomers, whose flexibility is dependent on the presence of N-terminal regions of the ectodomain (E1 domain and the acidic region).

CONCLUSION

Our results demonstrate that there are at least two A β species that bind to A β PP and produce unique changes in both oligomerization state and conformation: the 7-kDa species (which is most likely dimeric in solution) interacts with and monomerizes eA β PP, whereas a family of larger A β oligomers that is generated under conditions conducive to fibrillization of A β stabilizes the eA β PP dimer. The changes in both oligomerization state and conformation are associated with increased flexibility of the random coil regions of eA β PP, although this is not the type of conformational change typically thought to be induced by the binding of a ligand to a receptor. The observed changes fit well with the current understanding of A β PP's biology: for example, increasing the flexibility of the random coil regions increases the effective size of A β PP and allows A β PP (particularly the N-terminal domain) to search an increased volume of the cell-surface, thus enhancing the probability of ligand interaction. The GFLD domain in particular has been implicated in the binding of soluble trophic factors such as Reelin [54] and F-spondin [55], which have opposing effects on neurite retraction and outgrowth. Similarly, an extended A β PP is also more likely to function as an adhesion molecule, since it is more likely to find interaction partners on the opposing cell surface. Expansion also could potentially disrupt binding sites that depend on co-localization of the E1 and E2 domains. For example, both domains contain heparin binding sites [39]. Expansion could also expose new binding sites that are occluded by the association of the E1 and E2 domains.

Expansion should also affect the rate of proteolysis within both the acidic and A β -cognate regions. The relative flexibility of the acidic region might regulate access to the proteolysis site that generates N-A β PP. This is an N-terminal fragment of A β PP that contains the E1 domain and is a ligand for DR6 (death receptor 6), activating caspase-6 and inducing neurite retraction [13]. Interestingly, increasing the flexibility of the acidic region creates a tethered version of N-A β PP that is not present in either sA β PP α or the eA β PP₁₉₋₆₂₄ dimer because of the close association of the E1 and E2 domains. It should be pointed out that, although both A β -eA β PP complexes feature extended amino-terminal domains, with E1 and E2 dissociated, they most likely exist under different physiological conditions, since one is associated with A β species shown to be non-toxic, whereas the other is associated with toxic A β oligomers. It is the complex with the larger A β oligomers that is more likely to exist under the pro-AD conditions associated with an overproduction of A β , synaptic loss, and cell death.

Altering the rate of proteolysis at the α -cleavage site in the A β -cognate region could also have a direct impact on the overproduction of A β observed in AD. The relative flexibility of the A β -cognate region similarly may control access to the α - and β -cleavage sites. The relative rate of cleavage at these two sites is one of the factors that controls the rate of A β production. Although it is difficult to determine from our results whether binding of dimeric

A β would have a significant effect on α -cleavage, our results predict that the binding of moderately-sized soluble oligomers of A β should decrease α -cleavage through both stabilization of the A β PP dimer and restricted access to the cleavage site, as evidenced by the protection of K-612 in our crosslinking studies. This prediction is consistent with recent work showing that soluble A β oligomers decrease the production of sA β PP [22].

Finally, the results described herein suggest a mechanism through which binding of A β oligomers could alter A β PP function by sequestering the ectodomain of A β PP into homodimers. In addition, A β oligomers could potentially compete with proteins that interact with the ectodomain of A β PP to modulate signaling or A β PP processing. For example, competition between exogenously added A β_{1-42} , which rapidly aggregates into larger oligomers, and the common neurotrophin receptor p75^{NTR} for A β PP binding, has been demonstrated in cell culture [56]. This type of competition may have a significant impact on both the signaling and processing of A β PP, since the ectodomain of A β PP has been implicated in forming heterodimers and higher order complexes with a number of other ligands and receptors such as Netrin-1 [12], ApoE [57, 58], Notch [59, 60], p75^{NTR} [56], APLP1, APLP2 [61], BRI2 [62], and BRI3 [63]. For all of these proteins, interaction with A β PP occurs through a region containing the A β -cognate sequence. Although the functions of the British dementia proteins, BRI2 and BRI3, are unknown, the other proteins have been implicated in signal transduction complexes that regulate the balance of neurite outgrowth versus retraction. Our results suggest that increased concentrations of A β oligomers such as in early AD could potentially disrupt these interactions and therefore the delicate balance of neurite outgrowth and retraction that is critical to forming new memories.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Characterization of soluble A β species. A) Example of the size distribution of A β_{1-42} after 2 h of incubation at a concentration of 0.1 mg/ml (9 μ M) and 37°C. Three distinct size ranges were consistently observed, corresponding to 7-kDa, 20-kDa, and 70-kDa species. The A β_{1-42} was purified using a 24 ml Superdex S-75 column (GE Healthcare) with 10 mM phosphate pH 7.4, 137 mM NaCl (PBS) as the running buffer. The concentration of A β_{1-42} was monitored by the intensity of the absorbance at 280 nm. The column was calibrated with a Low Molecular Weight Calibration Kit (Sigma). B) Cross-linking analysis with BS3 showed that the 7-kDa pool consisted of mostly monomers and dimers, while the 20-kDa pool contained larger LDS-soluble oligomers that were inefficiently cross-linked to trimers. Lane 1 is 7-kDa A β_{1-40} with no BS3. Lane 2 is 20-kDa A β_{1-42} with 2.5 mM BS3. Lane 3 is 7-kDa A β_{1-40} with 2.5 mM BS3. The A β peptides at 0.1 mg/ml were incubated with 2.5 mM BS3 or PBS for 30 min at 24°C before analysis with denaturing gel electrophoresis (NuPAGE 4–12% gels, Invitrogen). The differential recognition of the trimer and monomer bands in the 6E10 Western blot indicates that the conformation of the A β is different between the 7-kDa and 20-kDa oligomers. The 6E10 epitope contains a lysine at position 612 (K-612) which can be modified by BS3. The absence of the monomer band in the 7-kDa A β_{1-40} indicates that K-612 is highly susceptible to BS3 modification. In contrast, the monomer band of the 20-kDa A β_{1-42} is still detectable, suggesting that some of the monomer results from disruption by LDS of larger oligomers in which K-612 is more protected from BS3 modification. C) A-11 positive A β_{1-40} was generated by incubating 0.1 mg/ml A β_{1-40} for 3 days at 37°C before purification using a 24 ml Superdex S-200 column (GE Healthcare) and PBS as the running buffer. The concentration of A β_{1-40} was monitored by the intensity of the absorbance at 280 nm. The dot blot in the inset shows the reactivity of 10 ng of the fraction indicated in C to 4 G8 and A-11. D) Cross-linking analysis with

BS3 of the indicated fraction. This pool also contained LDS soluble oligomers (lane 1, no BS3) close to 60-kDa in relative molecular mass (lane 2, +BS3).

Fig. 2.

Domain structure of eAβPP₁₉₋₆₂₄ and its fragments. The complete ectodomain of AβPP, referred to here as eAβPP₁₉₋₆₂₄, starting at the C-terminus of the signal sequence (residue 19 of AβPP) and ending at lysine residue 624. The domains are depicted as follows: thioredoxin (TRX) = tan box, growth factor ligand domain (GFLD) = orange box, Cu-binding (CuBD) = orange box, acidic region = teal line, RERMS = blue box, core AβPP domain (CAPPD) = blue box, unstructured region (residues 575–595) = pink line, Aβ cognate region (residues 596–624) = pink box. The cleavage site that creates N-AβPP and sAβPP_α are indicated with scissors. The exact location of the cleavage site within the acidic domain that creates N-AβPP is unknown as indicated by the question mark. Structures for fragments of AβPP containing the GFLD, CuBD, and RERMS-CAPPD domains have been determined by both x-ray crystallography and NMR. An alternate naming system groups the GFLD and the CuBD into the E1 domain and the RERMS-CAPPD domains into the E2 domain. Both the acidic region and residues 575–624 are predicted to adopt random coil conformations [64]. The Aβ-cognate region is shown as a box to distinguish it from the preceding residues. The composition of each eAβPP fragment and its molecular weight are shown.

Fig. 3.

Reconstruction of the shape of eAβPP₁₉₋₆₂₄ dimer from SAXS data using BUNCH. BUNCH is a modeling technique that models the protein as domains connected by a flexible chain. For all models, the domains were modeled using the X-ray crystal structures: thioredoxin (46), GFLD (47), Cu-binding domain (48), and RERMS-CAPPD domains (24). Both the acidic region (cyan or teal) and residues 575–624 (pink or dark red) are modeled as extended chains of balls. The E1 domains are orange or tan. The E2 domains are blue or light blue. This model, in which the chain folds to fill a defined volume, is consistent with the globular shape predicted by the Kratky plot (Fig. 7). Achieving an excellent fit to the data ($\chi = 1.5$, $(0.013 < q < 0.3)$) with a single conformation suggests that flexibility of both the acidic region and residues 575–624 are constrained within the eAβPP₁₉₋₆₂₄ dimer. Q is proportional to the scattering angle. The maximum value defines the resolution of the model that is 20 Å in this case. The fit between the observed scattering data and the scattering predicted from the model is shown in Supplementary Fig. 3. Thioredoxin was included in this model; however, it has been omitted from the figure for clarity and to emphasize the similarity of the monomer (top left) with the model for sAβPP [40]. The dimer is shown on the right in three different orientations to demonstrate the intermolecular contacts between the E2 domains and the 575–624 regions. The arrows in the right middle and right bottom images indicate the orientation of the E2 domain within each monomer. Because of the resolution of the model, the exact residues that participate in the contacts are undefined. Although the relative position of the E1 and E2 domains is well determined, the exact location of the residues in the 575–624 region cannot be determined because they all contribute the same subvolume and there are multiple ways to fold the two chains to create the subvolume. Therefore, the C-terminal tails of each molecule cannot be distinguished. This region is colored two different shades to indicate that the C-terminal tails of the molecules come together and create a dimer interface. A schematic (bottom left) serves both as a color key

and to show via the dashed lines the approximate locations of the two dimer interfaces. In this model, the thioredoxin domains and their flexible linker extend outward from the GFLD domain and do not interact with either monomer of eA β PP₁₉₋₆₂₄.

Fig. 4.

Aβ-binding is localized to residues 575–624 in eAβPP. A) Binding of A-11 positive 70-kDa Aβ₁₋₄₀ to eAβPP. 1.2 μM eAβPP₁₉₋₆₂₄ incubated with the A-11 positive 70-kDa pool of Aβ₁₋₄₀ oligomers shown in Fig. 1C for 1 h on ice. The elution profiles at 280 nm of 1.2 μM eAβPP₁₉₋₆₂₄ alone (blue), 1.2 μM eAβPP₁₉₋₆₂₄ incubated with a seven-fold molar excess of aged 70-kDa Aβ₁₋₄₀ (pink). The running buffer is 20 mM Tris pH 7.0, 100 mM NaCl for both columns. Western blot analysis with the 6E10 antibody (Covance) on the fractions illustrated by the shaded bars (11.9 mls-complex, 12.4 mls –eAβPP₁₉₋₆₂₄) indicates that the complex peak contains Aβ₁₋₄₀ as well as eAβPP₁₉₋₆₂₄. The inset dot blot on the complex fractions indicates that 6E10 reactivity is only found within the two peaks. Purified A-11 positive 70-kDa Aβ₁₋₄₀ incubated on ice for 1 hr at the same concentration (8.4 μM) eluted at 14.5 ml as in the SEC profile shown in Fig. 1C. The excess Aβ₁₋₄₀ elutes at the position expected for 7-kDa Aβ₁₋₄₀. B) Binding of 7-kDa Aβ₁₋₄₀ to eAβPP. eAβPP₁₉₋₆₂₄ or eAβPP₅₇₅₋₆₂₄ was incubated with 7-kDa Aβ₁₋₄₀ at a 1:20 molar ratio on ice for 1 h. Complexes were purified using a 24 ml Superdex S-200 column with a running buffer of 20 mM Tris pH 7.4, 50 mM NaCl. Fractions containing either eAβPP₁₉₋₆₂₄ or eAβPP₅₇₅₋₆₂₄ were analyzed by reducing SDS-PAGE for Aβ₁₋₄₀ content. The Aβ₁₋₄₀ was visualized using Sypro Ruby staining. The representative fractions shown are eAβPP₅₇₅₋₆₂₄ + Aβ₁₋₄₀ eluting at 17.8 ml (1), eAβPP₅₇₅₋₆₂₄ eluting at 15.5 ml (2), eAβPP₁₉₋₆₂₄ + Aβ₁₋₄₀ eluting at 12 ml (3), eAβPP₁₉₋₆₂₄ eluting at 10.8 ml (4). Purified 7-kDa Aβ₁₋₄₀ incubated on ice for 1 h at 1.2 μM eluted from the Superdex S-75 column as a single peak at 18 ml similar to the peak shown in Fig. 1A, and eluted from the Superdex 200 at 19.5 ml, similar to the excess Aβ₁₋₄₀ peak shown in A. The column profile for eAβPP₁₉₋₆₂₄ is analogous to the SEC profile shown in Supplementary Fig. 2B for an Aβ₁₋₄₂ pool dominated by 7-kDa Aβ₁₋₄₂.

Fig. 5.

Binding of eAβPP₁₉₋₆₂₄ to different species of Aβ. A) Size of the complex obtained by mixing eAβPP₁₉₋₆₂₄ with Aβ oligomers of varying sizes, as determined by size exclusion chromatography. eAβPP₁₉₋₆₂₄ incubated with a 20-fold molar excess of purified 7-kDa Aβ oligomers. The estimated molar excess was approximately 5-fold for the 20-kDa Aβ and 70-kDa Aβ. The volume of each incubation was adjusted so that the final concentration of the Aβ oligomers was approximately 10 μM and the final concentration of the eAβPP₁₉₋₆₂₄ was 0.6 μM. Samples were incubated on ice for 1 h. All of the 7-kDa Aβ pools were equally effective in reducing the apparent size of eAβPP₁₉₋₆₂₄ independent of the method by which the pool was made or the length of the Aβ peptide. To simplify the figure, only the results for Aβ₁₋₄₀ are shown for the 7-kDa species. The A-11 positive 70-kDa Aβ contains results for two Aβ₁₋₄₀ batches and two Aβ₁₋₄₂ batches since there was no significant difference between them. The A-11 negative contains results for three Aβ₁₋₄₀ batches. The elution volumes (V) were obtained using a Superdex S-200 24 ml column calibrated with a High Molecular Weight Calibration Kit (Sigma). The void volume (V₀) was determined using dextran blue. The running buffer was 20 mM Tris pH 7.4, 50 mM NaCl, 2.5 mM EDTA. The error bars reflect the variance obtained from making the complex with three (except where otherwise indicated) independent batches of Aβ-eAβPP₁₉₋₆₂₄ complexes for each type of Aβ oligomer. In both A and C, *** $p < 0.025$, ** $p < 0.05$, and * $p < 0.10$. The dotted lines mark the elution volume of the eAβPP₁₉₋₆₂₄ dimer (D) and the eAβPP₁₉₋₆₂₄ monomer (M) as verified with SAXS. B) Size and homogeneity of the complex obtained from mixing eAβPP₁₉₋₆₂₄ with Aβ oligomers of varying sizes as determined by SAXS. Guinier plot of eAβPP₁₉₋₆₂₄ (blue) and its SEC purified complex with A-11 negative 70-kDa Aβ₁₋₄₀ (purple), A-11 positive 20-kDa Aβ₁₋₄₂ (black), A-11 negative 26-kDa Aβ₁₋₄₀ (green) and 7-kDa Aβ₁₋₄₀ (red). The y-axis is normalized so that

one unit is proportional to 82-kDa (the molecular mass of eA β PP₁₉₋₆₂₄). The molecular mass of the complex can be obtained from the extrapolated y-intercept of the best-fit line. For clarity, the 26-kDa line was shifted down by 0.1 units. Plotted at its actual intercept of 2.1, it overlaps the 70-kDa line. The radius of gyration is proportional to the slope. The linearity of the data indicates that the samples are homogeneous in size and shape. C) Monomeric fragments of A β compete with eA β PP₁₉₋₆₂₄ dimerization. 0.6 μ M eA β PP₁₉₋₆₂₄ incubated with a 20-fold molar excess of A β peptides. The A β fragment peptides were dissolved as recommended by the manufacturer (AnaSpec) to a concentration of 100 μ M and their concentration was verified with the Pierce Coomassie Protein Assay Kit. The 7-kDa A β ₁₋₄₀ and A β ₁₋₄₂ peptides were purified with a Superdex 75 column prior to the incubation as shown in Fig. 1A. The elution volume and the void volume were determined as in A.

Fig. 6.

Only moderate sized soluble Aβ oligomers protect lysine K-612 from modification with BS3. A) Western blot of eAβPP₁₉₋₆₂₄ cross-linked with BS3 in the presence of dimeric Aβ₁₋₄₀ and A-11 positive 70-kDa Aβ₁₋₄₀ at a 1:5 mass ratio in PBS. Sypro Ruby staining detected both monomeric and dimeric eAβPP bands in all lanes containing cross-linked samples. B) Location of the epitope of different antibodies to the Aβ-cognate region with respect to BS3-modifiable lysines. The only lysine that is local to all three epitopes is K-612. C) Relative immunoreactivity of eAβPP₁₉₋₆₂₄ monomer after BS3 modification in the presence and absence of Aβ. The relative immunoreactivity was calculated as the ratio of the intensity of the monomer band in the presence of Aβ to its immunoreactivity in the absence, minus 1. Zero therefore represents no difference, while 2.0 corresponds to a three-fold difference. The bands were integrated with ImageJ. Coomassie staining was used as a loading control. The exact epitopes of the monoclonal antibodies 5A3 and 1 G7 are unknown, but they are both between residues 290–574, well outside of the Aβ cognate region. The results of cross-linking the 70-kDa Aβ₁₋₄₀, 20-kDa Aβ₁₋₄₂, and 7-kDa Aβ₁₋₄₀ in the absence of eAβPP₁₉₋₆₂₄ are shown in Fig. 1B and Fig. 1D. ***p* < 0.05 and **p* < 0.1.

Fig. 7.

Conformational changes in $eA\beta PP_{19-624}$ due to binding of $A\beta$ species. A) Examples of typical Kratky plots. The globular example is from SAXS data measured for bovine serum albumin at 1 mg/ml. The rod-shaped and compact domains connected by random coil are drawn from examples in Kratky and Glatter [51]. Aggregation of a protein can also produce a Kratky plot similar to the one labeled “compact domains connected by random coil.” Aggregation can be detected by a large increase in apparent molecular weight and non-linear Guinier plots. None of our samples showed this characteristic pattern (Fig. 5B). B) Kratky plot for $eA\beta PP_{19-624}$: alone (blue), in complex with 7-kDa $A\beta_{1-40}$ (red), and 20-kDa $A\beta_{1-42}$ (black). C) Kratky plots for $eA\beta PP_{290-624}$: alone (blue), 7-kDa $A\beta_{1-40}$ and 20-kDa $A\beta_{1-42}$.

Fig. 8.

Schematic showing the differential structural effects on the ectodomain of AβPP of binding monomeric Aβ peptides versus moderate-sized Aβ oligomers. The arrows indicate increased flexibility within the region. The AβPP domains are colored as E1 (orange), acidic region (cyan), E2 (blue), residues 575–595 (pink), Aβ cognate region (red), and cytoplasmic domain (green). In the diagram, we have shown the AβPP molecules breaking into monomers upon binding of monomeric/dimeric Aβ_{1–40}, as suggested by our results with binding of the monomeric helical Aβ fragments. However, residues within the transmembrane region have been shown to stabilize AβPP homodimers, as well (50). Similarly, a variety of adaptor proteins bind the AβPP cytoplasmic domain and could influence the stability of the homodimer (39). Furthermore, extracellular factors may also influence the homodimer-monomer equilibrium: for example, sAβPP is more efficient at disrupting AβPP homodimers in the presence of heparin (26). Undoubtedly, the efficiency of monomeric/dimeric Aβ_{1–40} in completely separating the AβPP homodimers will be modulated by additional factors *in vivo* (n is the number of Aβ peptides bound to AβPP that is consistent with the SAXS results).

Table 1

SAXS Analysis of eAβPP fragments and their complexes with Aβ peptides

	Concentration (mg/ml)	Peptide	Aβ Size (SEC)	R _g (Å) (±2)	D _{max} (Å) (±5Å)	Relative Mass (±0.2)	Aβ Detected
sAβPP _α				42	135		
eAβPP ₁₉₋₆₂₄ (82-kDa)	0.8	na	na	60	200	2.0	na
	0.72	1-40	7-kDa	50	170	1.2	WB
	0.87	1-40	20-kDa*	58	200	2.4	SR
	0.78	1-40	26-kDa	57	200	2.1	SR
	1.4	1-40	70-kDa	58	200	2.1	WB
	0.71	1-42	20-kDa*	56	200	2.3	WB
eAβPP ₂₉₀₋₆₂₄ (52-kDa)	1.2	na	na	41	150	2.1	na
	0.4	1-40	7-kDa	40	140	1.1	WB
	1.2	1-40	20-kDa*	40	140	2.3	WB
	1.3	1-42	20-kDa*	43	160	2.5	WB
eAβPP ₂₃₀₋₆₂₄ (45-kDa)	1.2	na	na	43	155	2.0	na
	0.5	1-42	20-kDa*	44	155	2.2	WB
eAβPP ₅₇₅₋₆₂₄ (20-kDa)	1.0	na	na	32	100	2.6	na

The radius of gyration (R_g) and the maximum dimension (D_{max}) were obtained using the program GNOM. The relative mass (ON) was calculated as the ratio of the apparent mass of the protein to the expected mass derived from the protein sequence (M). For globular, non-interacting proteins, the apparent mass can be estimated by comparing the extrapolated scattering of the sample at zero scattering angle ($I(0)$) to that of a reference protein (bovine serum albumin, ovalbumin, yeast alcohol dehydrogenase or carbonic anhydrase (Sigma)) with the equation; $ON = (C_{ref}M_{ref}I_{un}(0))/(C_{un}M_{un}I_{ref}(0))$; where subscripts *un* and *ref* refer to the sample and the reference protein [26]. Reference proteins of different sizes were used in this study to eliminate biases due to protein size. All calculations fell into the range of ± 0.2 of the number reported in Table 1 for each reference protein. This calculation overestimates the apparent mass for proteins with extensive regions of random coil. Monomeric proteins with 20-30% of their residues in random coil conformation, as expected for monomeric AβPP, have relative mass estimates around 1.3⁵¹. To confirm the presence of Aβ in the complexes, samples were analyzed by SDS-PAGE. A band corresponding to monomeric Aβ₁₋₄₀ was detected with Sypro Ruby staining (SR) or Western blotting (WB) with the 4 G8 antibody.

* The next to the molecular weight indicates oligomers that were A-11 positive. Complexes formed by incubation of eAβPP fragments with 7-kDa Aβ₁₋₄₂, A-11 positive 70-kDa Aβ₁₋₄₀, and A-11 positive 70-kDa Aβ₁₋₄₀ were not analyzed by SAXS because of difficulties in producing sufficient material for the experiments. The radius of gyration (R_g) and maximum dimension (D_{max}) for sAβPP_α were obtained from Gralle et al. [40].