

Apolipoprotein E includes a binding site which is recognized by several amyloidogenic polypeptides

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Inheritance of the apolipoprotein E (apoE) $\epsilon 4$ allele is a risk factor for late-onset Alzheimer's disease (AD). Biochemically apoE is present in AD plaques and neurofibrillary tangles of the AD brain. There is a high avidity and specific binding of apoE and the amyloid β -peptide (A β). In addition to AD apoE is also present in many other cerebral and systemic amyloidoses, Down's syndrome and prion diseases but the pathophysiological basis for its presence is still unknown. In the present study we have compared the interaction of apoE with A β , the gelsolin-derived amyloid fragment AGel_{183–210} and the amyloidogenic prion fragments PrP_{109–122} and PrP_{109–141}. We show that, similar to A β , also AGel and PrP fragments can form a complex with apoE, and that the interaction between apoE and the amyloidogenic

protein fragments is mediated through the same binding site on apoE. We also show that apoE increases the thioflavin-T fluorescence of PrP and AGel and that apoE influences the content of β -sheet conformation of these amyloidogenic fragments. Our results indicate that amyloids and amyloidogenic prion fragments share a similar structural motif, which is recognized by apoE, possibly through a single binding site, and that this motif is also responsible for the amyloidogenicity of these fragments.

Key words: Alzheimer's disease, amyloidosis of the Finnish type, pathological chaperone, prion disease.

INTRODUCTION

Amyloidoses are a group of disorders characterized by the abnormal extracellular deposition of insoluble amyloid fibrils, consisting usually of soluble proteins or fragments thereof, in one or several tissues (for a review, see [1]). Although varying in their origins, the disease-state fibrils all share certain properties that include an apple-green birefringence when viewed under polarized light after Congo Red staining, a predominantly β -pleated sheet secondary structure, a typical fibrillar appearance seen in electron microscopy, and a high degree of insolubility under physiological conditions. Arrays of prion rods found in several prion disorders resemble amyloid ultrastructurally and share similar properties [2,3].

Alzheimer's disease (AD), the most common of the amyloidoses, is characterized pathologically by extracellular deposits of a fibrillar 39- to 43-amino acid-long amyloid peptide [4]. This peptide, called Alzheimer's amyloid β -peptide (A β), is derived by an as-yet unknown proteolytic pathway of an integral membrane protein called amyloid precursor protein (APP; for a review, see [5]). A β is thought to play a crucial role in the pathogenesis of AD because several mutations in the APP gene lead to the early-onset form of the disease (for a review, see [6]). Despite the fact that genetic mutations have been found to cause the disease [7–9], a vast majority seem to be sporadic cases.

Prion-related disorders (spongiform encephalopathies) are characterized by intra- and extracellular accumulation of an abnormal isoform of the cellular prion protein (PrP^c) called scrapie prion protein (PrP^{Sc}). The pathological form of the PrP and its cellular precursor is expressed in the brain and also, at

lower concentrations, in peripheral tissues. Prion diseases in human include Kuru, Creutzfeldt–Jakob disease, Gerstmann–Straussler–Scheinker syndrome and fatal familial insomnia (for a review, see [10]). Similar to AD, prion diseases occur both as sporadic and autosomal dominant familial diseases.

Familial amyloidosis of the Finnish type (FAF) is characterized by extracellular deposition of abnormal fibrillar material derived from gelsolin, an actin-modulating protein. So far, FAF has been found to be caused by only two nucleotide substitutions, changing Asp¹⁸⁷ to Asn¹⁸⁷ or Tyr¹⁸⁷ (for a review, see [11]), resulting in the inactivation of one of the two actin-binding domains in gelsolin [12]. The mutation leads to a misfolded protein, which becomes predisposed to an as-yet-unknown degradation pathway, finally leading to the formation of an abnormally processed amyloid peptide fragment (AGel, gelsolin-derived amyloid of the Finnish type) [13]. No sporadic cases of FAF are known.

In prion diseases and amyloidoses, the protein or a fragment thereof undergoes conformational changes involving a shift from α -helix to β -sheet. It is likely that these conformational changes are crucial for the propagation of the disease (for a review, see [14]). How such changes occur is still largely unknown.

Apolipoprotein E (apoE), a 34-kDa protein coded by a gene on chromosome 19, is produced by a polymorphic gene producing three major isoforms, E2, E3 and E4. These isoforms are coded by three *apoE* gene alleles, $\epsilon 2$, $\epsilon 3$ and $\epsilon 4$, of which the $\epsilon 4$ isoform has generated considerable interest due to genetic evidence suggesting a link between this particular apolipoprotein and the pathogenesis of late-onset AD [15–18], Lewy body dementia [19], dementia in Parkinson's disease [20] and dementia and cognitive performances in Down's syndrome patients [21,22]. Immuno-

Abbreviations used: A β , A β _{1–40}, Alzheimer's amyloid β -peptide; AGel, gelsolin-derived amyloid of the Finnish type; PrP, prion protein; AD, Alzheimer's disease; APP, Alzheimer's amyloid precursor protein; FAF, familial amyloidosis of the Finnish type; apoE, apolipoprotein E; ThT, thioflavin-T.

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logically, apoE is present in many cerebral and systemic amyloidoses, Down's syndrome and prion disorders. Recent studies support the concept that apoE would directly be involved in the amyloid deposition and fibril formation [15,17,23,24]. Complete lack of apoE has been shown to dramatically reduce A β and amyloid A deposition in transgenic mice overexpressing the APP^{V717F} mutation [25] or amyloid A, respectively [26]. Moreover, expressing human apoE instead of the mouse apoE in an AD mouse model results in a even more dramatic reduction of A β deposition [27]. These experiments indicate clearly a crucial role for this specific apolipoprotein in amyloid formation.

In the present paper we have compared four different amyloidogenic fragments, A β ₁₋₄₀, PrP₁₀₉₋₁₂₂ and PrP₁₀₉₋₁₄₁ and AGel₁₈₃₋₂₁₀ for their interaction with human apoE. Here we show that, comparable with A β ₁₋₄₀, also PrP and AGel fragments can form SDS-stable complexes with apoE. Complex formation is strongest with the AGel fragments. Similar to A β ₁₋₄₀ and AGel, apoE seems to also enhance the amyloidogenicity of the PrP fragments. Moreover, all three amyloid fragments compete for the same binding site on apoE.

To verify the unique binding properties of apoE we have performed similar experiments with a non-amyloid-associated protein, amphoterin. Amphoterin has a high capacity to bind amyloidogenic peptide fragments, but the binding properties of amphoterin are, however, completely different from those observed with apoE.

Our findings suggest that amyloids and prions share a limited structural motif which, after induction, can accelerate the adoption of a β -sheet conformation and that this motif is the same as that recognized by apoE, evidently through a common binding site. Our findings provide a possible explanation for the widespread accumulation of apoE in these diseases.

MATERIALS AND METHODS

Materials

A β ₁₋₄₀ (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV), gelsolin amyloid AGel_N (FNNGDCFILDLGNNIHQWCGSNSNRYER), AGel_D (FNNGNCFILDLGNNIHQWCGSNSNRYER) and AGel_Y (FNNGYCFILDLGNNIHQWCGSNSNRYER), corresponding to amino acids 183–210 of human plasma gelsolin [28], where N, D and Y indicate the amino acid present at position 187 of the whole protein and position 10 of the peptide, were synthesized at the Centre for the Analysis and Synthesis of Macromolecules (SUNY, Stony Brook, NY, U.S.A.). Prion peptides PrP₁₀₉₋₁₂₂ (PrP-H1; MKHMAGA-AAAGAVV) and PrP₁₀₉₋₁₄₁ (PrP-H1H2; MKHMAGAAAAG-AVVGGLGGYMLGSAMSRPMMHF) were purchased from Bio-Synthesis (Lewisville, TX, U.S.A.). All peptides were purified by microbore HPLC. The sequence and purity of each peptide was verified by matrix-assisted laser desorption (MALDI) MS and/or automated Edman degradation. Stock solutions of each peptide (2 mg/ml) were prepared in 50% acetonitrile in 0.1% trifluoroacetic acid in water and stored at -70°C . Human plasma apoE and the recombinant apoE4 isoform, produced by the eukaryotic baculovirus expression system, were purchased from Calbiochem-Novabiochem (Läufelfingen, Switzerland) and PanVera Corp. (Madison, WI, U.S.A.), respectively. The proteins were found to be more than 95% pure by silver staining on SDS/PAGE. Antibodies against A β (4G8) recognizing the 17–24 fragment were purchased from Senetek (St. Louis, MO, U.S.A.). Recombinant amphoterin was received as a kind gift from Dr H. Rauvala (Institute of Biotechnology, University of Helsinki, Helsinki, Finland). The protein was found to be more than 95% pure by silver staining on SDS/PAGE.

Co-incubation experiments with apoE

Purified apoE or recombinant apoE4 (2 μg each, 0.72×10^{-10} M) were incubated at 37°C for 16 h with A β (1–30 μg , 0.025 – 0.75×10^{-8} M) and/or with AGel_{D/N/Y} (1–20 μg , 0.022 – 0.44×10^{-8} M) and/or PrP₁₀₉₋₁₄₁ (1–30 μg , 0.023 – 0.69×10^{-8} M) in 50 μl of PBS, pH 7.2. Aliquots of the peptide stock solutions were lyophilized and mixed with the desired amount of the apolipoprotein. Formed complexes were analysed by high-resolution analytical Tris/Tricine SDS/PAGE. The solubility of each peptide was tested after lyophilization by Tris/Tricine SDS/PAGE and microbore gel-filtration liquid chromatography (SMART System, Pharmacia Biotech, Uppsala, Sweden).

Competitive-inhibition experiments with amphoterin

Purified recombinant amphoterin (2 μg , 0.7×10^{-10} M) was incubated at 37°C for 16 h with increasing amounts of A β (1–30 μg ; 0.025 – 0.75×10^{-8} M) and/or with AGel_{D/N/Y} (1–20 μg , 0.022 – 0.44×10^{-8} M) in 50 μl of PBS, pH 7.2. Formed complexes were analysed by high-resolution analytical Tris/Tricine SDS/PAGE.

Competitive-inhibition experiments with apoE and apoE4

apoE and apoE4 (2 μg , 0.72×10^{-10} M) were incubated at 37°C for 16 h with increasing amounts of A β (1–20 μg , 0.025 – 0.5×10^{-8} M) and/or with AGel_{D/N/Y} (1–20 μg , 0.022 – 0.44×10^{-8} M) and/or PrP₁₀₉₋₁₄₁ (1–20 μg , 0.023 – 0.46×10^{-8} M) in 50 μl of PBS, pH 7.2. The samples were analysed by high-resolution analytical Tris/Tricine SDS/PAGE.

Tris/Tricine SDS/PAGE

The protein–peptide complexes were analysed by Tris/Tricine SDS/PAGE according to Schaeffer and von Jagow [29]. Incubations were stopped by the addition of a modified Laemmli sample buffer (without β -mercaptoethanol and only 50% of the recommended SDS added) into each sample vial. Samples were not boiled but incubated at 37°C for 5–10 min. Protein–peptide complexes were electrophoresed on Tris/Tricine gels (10–14.5% T/3% C, with a spacer gel of 10% T/3% C; where T is acrylamide and C is cross-linker bisacrylamide). Protein bands were visualized by Coomassie Brilliant Blue or silver staining.

Fluorometric experiments

For the fluorometric experiments, approx. 30 μg (0.66×10^{-8} M) of each of the AGel_{D/N/Y} peptides or 30 μg (0.75×10^{-8} M) of A β or 30 μg (0.33×10^{-8} M) of PrP₁₀₉₋₁₂₂ was incubated with apoE in a ratio of 1:200 (mol lipoprotein/mol peptide) in 50 μl of PBS, pH 7.2, for 0–96 h. Incubated samples were then added to 50 mM glycine, pH 9, with 2 μM thioflavin-T (ThT; Sigma, St. Louis, MO, U.S.A.) in a final volume of 2 ml. Fluorescence was measured at the excitation and emission maxima of 435 and 485 nm, respectively, in a Hitachi F-2000 fluorescent spectrophotometer. A time scan of fluorescence was performed and three values obtained after the decay reached a plateau (mostly at 300–400 s) were averaged after subtracting the background fluorescence of the ThT and buffer alone. Two identical samples were measured. The PrP₁₀₉₋₁₄₁ peptide could not be used in the ThT assay due to a high background variation.

CD spectroscopy

CD spectra were obtained with a JASCO spectropolarimeter J-720 at room temperature in a 0.1-cm path-length cell. Double-

distilled water, 100 mM Tris/HCl, pH 7.2, and trifluoroethanol (spectroscopic grade) were used as solvents. Spectra were recorded at 1-nm intervals over the wavelength range 190–260 nm. All spectra were obtained by subtracting the buffer baseline and smoothed by using the algorithm by JASCO. The β -sheet conformation was determined following the relative change of the ellipticity at 217 nm of each identical pair of samples.

Densitometry

Each gel was scanned by a Hewlett-Packard Scanjet 4C laser scanner and each band of interest subsequently analysed by the Scion Image Program (SIP, Scion Corp., Frederick, MA, U.S.A.).

Western-blotting analysis

Western-blot analyses were performed to gels prepared as described in the section on competitive-inhibition experiments (see above). SDS/PAGE samples were transferred on to PVDF membranes. Immunoblotting was performed with anti-Alzheimer's β -amyloid peptide monoclonal antibodies (4G8). Membranes were blocked for 1 h with 3% BSA, washed, and then incubated with the primary antibody for 2 h. This was followed by washing and incubation with alkaline phosphatase-conjugated secondary antibody (anti-mouse antibody; Bio-Rad, Richmond, CA, U.S.A.). The staining was carried out according to the manufacturer's instructions.

Surface-plasmon-resonance spectroscopy

Surface-plasmon-resonance spectroscopy measurements were made with a BIACore 2000TM instrument (BIACORE, Pharmacia Biotech) using a CM5 sensor chip and the manufacturer's recommendations for amine coupling. Ligand levels were used that gave a clearly positive signal for the analyte binding. $A\beta$ and AGel_N were immobilized on the sensor chip, each in one channel. The running buffer (PBS) was applied at a flow rate of 20 μ l/min. apoE (150 pM solution) alone, apoE (150 pM) with $A\beta$ (1:10, apoE/ $A\beta$ molar ratio), and apoE (150 pM) with glycine (1:200, apoE/Gly molar ratio) were used as analytes. The measurements were conducted at 37 °C. The binding response of each sample solution was determined by the measurement of resonance signal units. Prior to the numerical analysis, data were adjusted to zero immediately before injection of analyte.

RESULTS

All synthetic peptides used in this study have previously been shown to be amyloidogenic [3,4,30]. Based on the fact that AD and prion disorders occur both as familial and sporadic forms, we decided to include the non-mutated form of the FAF amyloid into this study, although no sporadic cases of this disease are known. The non-mutated FAF peptides seem to be able to adopt a β -sheet structure similar to that of the mutated peptides (0.0% α -helix, 86.7% β -sheet) according to the predicted secondary structure by Chou and Fasman [31] and Garnier et al. [32]. Indeed, when incubated under similar conditions to $A\beta$, the non-mutated peptides also form amyloid fibrils indistinguishable from those formed by the mutated FAF amyloid fragments [24,33].

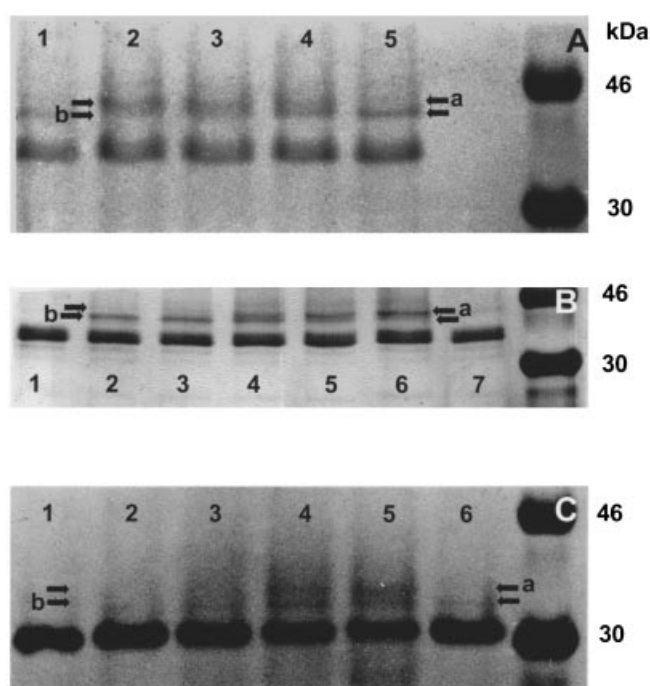


Figure 1 Coomassie Brilliant Blue-stained 16% Tris/Tricine polyacrylamide gels of (A) human plasma apoE, (B) apoE4 and (C) amphoterin (all 2 μ g), incubated for 16 h at 37 °C with AGel_N and $A\beta$

(A) Lane 1, apoE with 0.5 μ g of AGel_N; lane 2, apoE with 1 μ g of AGel_N and 20 μ g of $A\beta$; lane 3, apoE with 2 μ g of AGel_N and 15 μ g of $A\beta$; lane 4, apoE with 1 μ g of AGel_N and 10 μ g of $A\beta$; lane 5, apoE with 1 μ g of AGel_N. (B) Lane 1, apoE4; lane 2, apoE4 with 1 μ g of AGel_N; lane 3, apoE4 with 1 μ g of AGel_N and 5 μ g of $A\beta$; lane 4, apoE4 with 1 μ g of AGel_N and 10 μ g of $A\beta$; lane 5, apoE4 with 1 μ g of AGel_N and 15 μ g of $A\beta$; lane 6, apoE4 with 1 μ g of AGel_N and 20 μ g of $A\beta$; lane 7, apoE4 with 1 μ g of $A\beta$. (C) Lane 1, amphoterin; lane 2, amphoterin with 1 μ g of AGel_N and 1 μ g of $A\beta$; lane 3, amphoterin with 2 μ g of AGel_N and 5 μ g of $A\beta$; lane 4, amphoterin with 2 μ g of AGel_N and 10 μ g of $A\beta$; lane 5, amphoterin with 2 μ g of AGel_N and 15 μ g of $A\beta$; lane 6, amphoterin with 1 μ g of AGel_N. In all panels, a and b indicate complexes formed by each peptide (a, with $A\beta$; b, with AGel_N).

apoE forms SDS-stable complexes with PrP₁₀₉₋₁₄₁ and AGel

In order to compare the capability of apoE to form SDS-stable complexes with $A\beta$, AGel and PrP we incubated various amounts of these peptides under physiological conditions with apoE and apoE4. Incubation over 16 h resulted in the formation of SDS-stable complexes which could, in the case of AGel, be visualized by high-resolution Tris/Tricine SDS/PAGE as a faint band just above the major apoE band (Figure 1). Both PrP peptides turned out to be extremely difficult to visualize as a complex with apoE since they produced rather diffuse bands, probably due to the high tendency for aggregation of these particular peptides. The apoE–PrP band was almost on the same level with the native apoE, even with the 3.27-kDa PrP₁₀₉₋₁₄₄ peptide. Subsequently, we measured the apoE–PrP complex formation and binding competition between $A\beta$ and PrP to apoE by indirect Western-blot analysis, as shown in Figure 2. The mutated and non-mutated gelsolin peptides revealed a higher affinity to apoE as compared with the affinity of $A\beta$ or PrP. Densitometrical analyses indicated that the AGel peptides have an approx. 10-fold higher complex-forming affinity (Figure 3). $A\beta$ and the PrP fragments showed only moderate differences in their capability to form complexes with apoE (Figure 2).

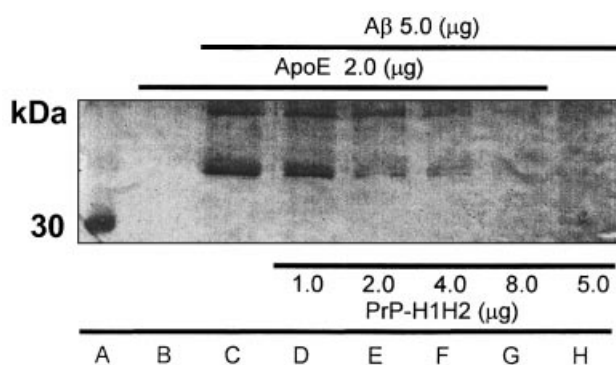


Figure 2 Western-blot analysis of apoE4 (2 μ g) with A β (5 μ g) and various amounts of PrP₁₀₉₋₁₄₁ (PrP-H1H2) incubated for 16 h at room temperature

The samples were blotted on to PVDF and visualized by a monoclonal anti-A β antibody (4G8) except lane A, which was molecular-mass standard (Coomassie Brilliant Blue-staining).

No differences could be detected in the binding of the mutated and non-mutated AGel peptides indicating that the mutation itself is not important for the binding. This is not surprising since none of the AGel mutations change the predicted β -sheet content

of these peptides, a factor seemingly crucial for the binding to apoE [34].

apoE influences the fibril formation of PrP and AGel fragments

The influence of apoE on the amyloid fibril formation of these peptides was monitored by the ThT method. ThT has been shown to bind to amyloid, producing a shift in the emission spectrum and a fluorescence signal proportional to the amount of amyloid formed [35]. This measurement was mainly conducted with the PrP₁₀₉₋₁₂₂ (PrP-H1) peptide due to a high background drift of the PrP₁₀₉₋₁₄₁ (PrP-H1H2), probably caused by the high-aggregation property of this particular peptide. We have reported earlier that apoE can accelerate the fibril formation of some non-mutated and mutated AGel peptide fragments *in vitro* and here we compared the effect of apoE on A β , AGel and PrP. Similar to our earlier observations [24], AGel_N, less amyloidogenic at pH 7.2, became highly amyloidogenic (increase approx. 4.5-fold) by the influence of this apolipoprotein. On the other hand, AGel_N, highly amyloidogenic at pH 7.2, was influenced less by apoE (increase approx. 1.3-fold), resembling the effect of apoE on the Dutch A β variant (A β _{1-40Q}; increase approx. 2-fold) [36]. Also the wild-type peptide AGel_D was clearly affected (increase approx. 1.6-fold). AGel_N is more amyloidogenic at pH 7.2 than AGel_V

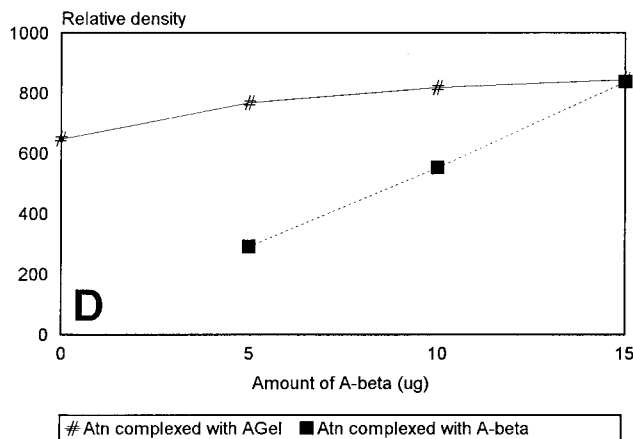
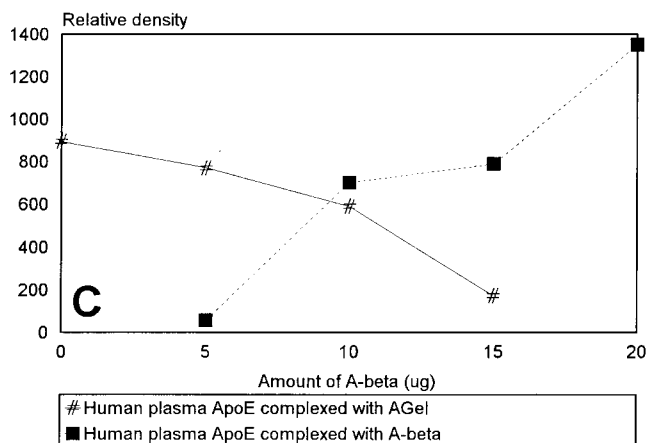
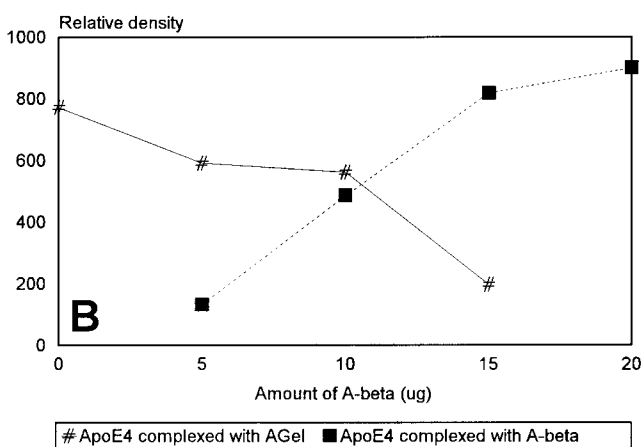
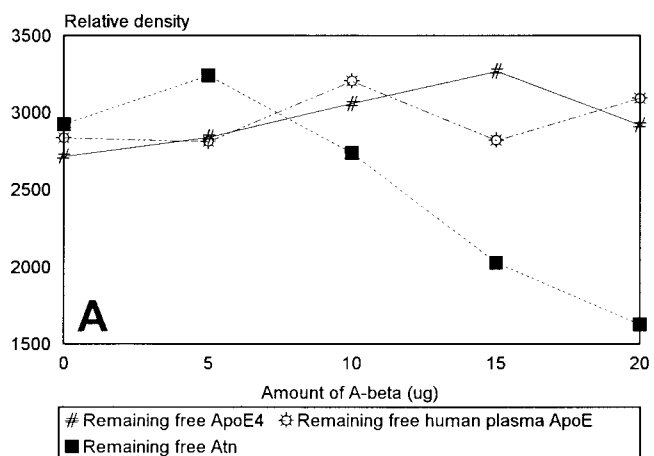


Figure 3 Densitometrical analyses of formed complexes, free apoE4, free human plasma apoE and free amphoterin (Atn) from Figure 1

(A) Band intensities of remaining free apoE4, free human plasma apoE and free amphoterin were measured after increasing the amount of A β from 0 to 20 μ g. (B) Band intensities of AGel_N and A β complexes with apoE4 after increasing the amount of A β from 0 to 20 μ g. (C) Band intensities of AGel_N and A β complexes with human plasma apoE after increasing the amount of A β from 0 to 20 μ g. (D) Band intensities of AGel_N and A β complexes with amphoterin after increasing the amount of A β from 0 to 15 μ g.

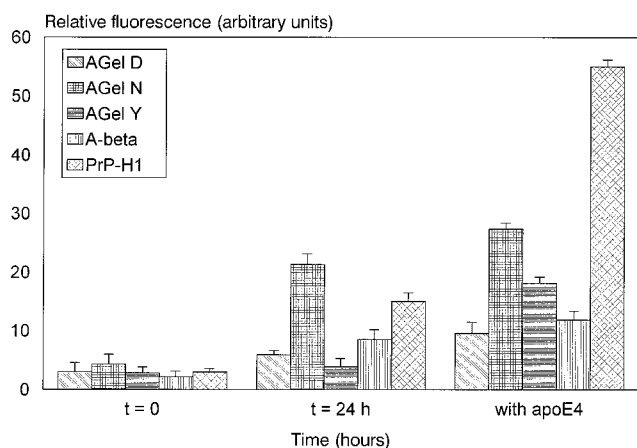


Figure 4 ThT assay of formed amyloid fibrils

Each sample was incubated for 16 h with or without human plasma apoE in duplicate (similar results were achieved with apoE4). Values are given in relative fluorescence (arbitrary units).

and therefore probably less influenced by any external promoters. These data indicate that a peptide which is able to form amyloid fibrils rapidly does not have much help from an 'external chaperone'. As expected, apoE also enhanced the fibril formation of the PrP fragment (increase approx. 3.5-fold), resembling that of AGel_Y (Figure 4).

A β , AGel and PrP compete for binding with apoE

The fact that A β , AGel and PrP were all similarly affected by apoE led us to study whether the effect of this apolipoprotein would be mediated through the same binding site on apoE. To do this we mixed various amounts of A β , AGel and PrP together with apoE and apoE4 for 16 h, after which time the formed complexes were analysed by Tris/Tricine SDS/PAGE (A β and AGel) and Western blotting (A β and PrP). When AGel and A β were incubated in a molar ratio of 1:1 no complex between A β and apoE was seen, only a complex between AGel and apoE could be detected; a 10-fold excess of A β resulted in an approx. 1:1 ratio of the formed complexes of AGel–apoE and A β –apoE and a 20-fold excess of A β washed out the AGel–apoE complex practically completely (Figures 1 and 2). The competition was independent of the peptide, e.g. each peptide could wash out the other. A molar ratio of approx. 2:1 of A β /PrP with apoE resulted in a 1:1 capability of complex formation, indicating that these two amyloidogenic peptides had only small differences in their complex-formation capacity with apoE under conditions used in this study (Figure 2). The formation or dissociation of the A β –apoE complex in the presence of AGel could be followed by Tris/Tricine SDS/PAGE, and the effect of PrP by Western-blot analysis using a monoclonal anti-A β antibody (Figure 2). No detectable differences in the complex-forming capacity between apoE and apoE4 were observed, indicating that different isoforms share the same binding. Human plasma apoE is a mixture of the three major isoforms, mainly composed of E3 (approx. 80%). It is worthwhile to note that experiments *in vivo* show no differences between the apoE isoforms in terms of their capability to form complexes with β -amyloid in human AD brain, e.g. each apoE isoform can form the same amount of complex *in vivo* [17,37]. In addition, there is no difference in the reduction of amyloid deposition between a mouse expressing human apoE3 or apoE4 compared with the corresponding mouse gene in an AD mouse

Table 1 Plasmon-resonance spectroscopy on the interaction and competition of A β and AGel_N with human plasma apoE

A β and AGel_N were bound to the matrix in one channel each. Responses were measured in real time with human plasma apoE, human plasma apoE with glycine (molar ratio 1:200) and human plasma apoE with A β (molar ratio 1:10) with the coupled ligands. Values are given as resonance signal units (in %, \pm S.D.). The resonance signal of apoE in each channel was set at 100%.

Ligand bound to the sensor surface	apoE	apoE + glycine	apoE + A β
A β	100 \pm 5.6%	83 \pm 10.9%	30 \pm 10.0%
AGel _N	100 \pm 9.5%	76 \pm 5.5%	37 \pm 5.7%

model [27]. None of these amyloidogenic peptide fragments bound to, or accelerated the amyloid fibril formation of, the other. Pre-incubating one peptide with apoE for several hours did not effect the competitive binding. Thus the binding of amyloid peptides to apoE seems to be reversible.

Verification of the competition by surface-plasmon-resonance spectroscopy

In order to verify that the competitive inhibition of the binding of A β and AGel to apoE is not artificially supported by the PAGE technique, we also studied the competitive inhibition by surface-plasmon-resonance spectroscopy. Both peptides (A β and AGel) were coupled to the chip and the affinity of the soluble apoE in the absence and presence of A β to bound A β and AGel measured. A 200-fold molar excess of glycine was used as a control. This high amount of glycine inhibited the apoE affinity to both peptides by approx. 20–30%, presenting the quasi-maximal physical inhibition of a molecule without any affinity to the ligand. An 8-fold molar excess of A β in the same solution with apoE inhibited the affinity of the free apoE to A β by approx. 70% and to AGel by approx. 60% (Table 1). Thus the presence of A β together with apoE in the same solution inhibited the affinity of the free apoE in that solution to the bound A β and also to the bound AGel, clearly supporting our gel-electrophoresis data. If A β would not bind to the soluble, native apoE through the same binding site as AGel, it should not have influenced the apoE–AGel interaction. These data verify that A β is indeed able to inhibit the apoE interaction with an unrelated amyloidogenic protein fragment like AGel. Due to its high tendency to aggregate we could not include any of the PrP fragments in these studies.

Control studies with amphoterin

To verify the reversibility and specificity of the binding of apoE and the amyloidogenic fragments, we have performed identical competition studies with a non-amyloid associated protein, amphoterin (high-mobility group-1 protein, HMG-1). This protein features the capability to bind amyloidogenic peptide fragments although it has not been found to be associated with any of the known amyloidoses. It has, however, the capacity to accelerate the amyloidogenicity of these peptides and includes a fragment that can form amyloid-like fibrils *in vitro*. In Figures 1 and 2 we show results of a competition experiment performed between AGel, A β and amphoterin. Increasing the amount of A β in a solution with amphoterin clearly decreased the amount of soluble free amphoterin as well (Figure 3A), indicating that the binding is irreversible under conditions used in this study. On the other hand, increasing the amount of A β in a solution with

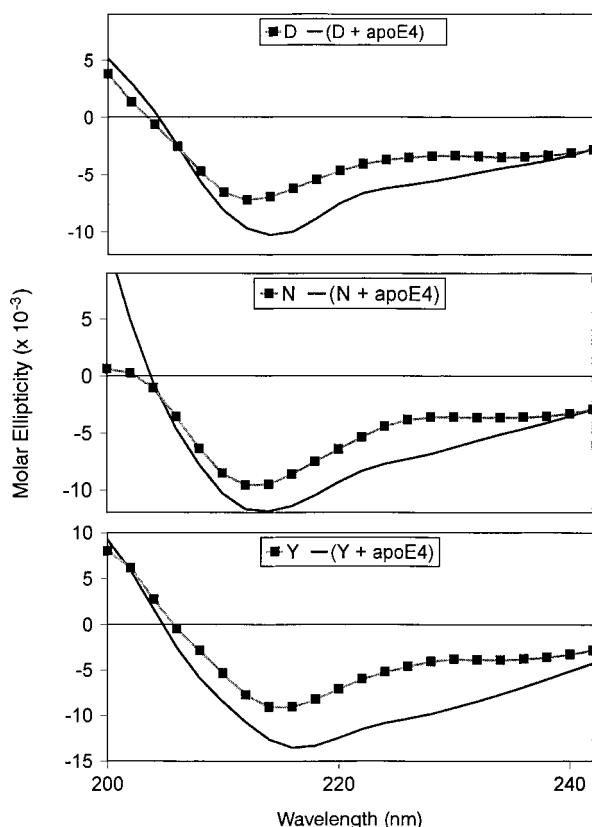


Figure 5 The effect of apoE4 on the AGel peptides as measured by CD

Each peptide (60 μ g) was dissolved in 60% trifluoroethanol/100 mM Tris/HCl (pH 7.2) and immediately applied to CD measurement ($t = 0$ h). apoE4 was added to each peptide solution in a final molar concentration of 1:200 (apoE/A β). Each measurement was repeated after incubation overnight at 37 $^{\circ}$ C ($t = 24$ h). The CD spectrum of apoE4 alone did not deviate from the buffer alone value due to the very low amount of protein used.

apoE did not reduce the amount of soluble apoE (Figures 1A, 1B and 2A). AGel and A β had similar effects on amphoterin and apoE (results not shown). In addition, none of the peptides competed for the binding with amphoterin, even at higher loads of one or the other (Figures 1C and 2D). Interestingly, increasing the amount of one of the peptides in a solution with amphoterin seems also to increase slightly the capacity of the other peptide to form a complex (Figure 1C and 3D). This could be due to two different binding sites, which upon single occupation can change the affinity of the other.

apoE influences the conformation of AGel

To determine whether apoE is able to induce a conformational change in the secondary structure of other amyloids, as it has been shown to do in A β [24], we have performed CD analyses of AGel peptides with and without apoE. Each peptide was dissolved in aqueous solution containing 60% trifluoroethanol, a condition which optimizes the α -helix formation but does not influence the β -sheet structure. Addition of apoE (apoE/AGel, molar ratio 1:200) had a specific effect on the spectrum with principal changes observed at wavelengths of 195 and 217 nm (Figure 5; β -sheet structure maximum at 195 nm and minimum at 217 nm), similar to results obtained with e.g. A β and apoE [34,38] or A β and aluminium [39]. CD spectrum of apoE alone

did not deviate from the buffer alone value due to the very low amount of protein used.

DISCUSSION

Recent genetic data suggest a heterogeneity among causes and risk factors for AD. These include mutations in the APP [7], presenilin 1 [8] and 2 [9], all responsible for the familial form of this disease, and a genetic association with the apoE ϵ 4 allele [16].

While a genetic mutation clearly leads to the disease, the role of apoE seems to be much more complicated. In fact, although many AD patients carry either one or two ϵ 4 alleles, it is not a prerequisite, since a number of AD cases do not show any linkage to this particular allele [40]. Moreover, the effect of apoE is not limited to AD. apoE-deficient mice overexpressing amyloid A show reduced amyloid fibril formation, as well [26]. In addition, deposits in various amyloidoses and prion diseases include both biochemically and immunohistochemically detectable amounts of apoE [17,23,41–43]. Thus the molecular interaction of apoE seems not to be specific for AD but more a common characteristic in the development of other amyloidoses as well.

We have shown previously that apoE can accelerate the amyloid fibril formation of A β by causing a conformational change of a non-pathogenic form of the amyloid peptide to a pathogenic form of the same peptide [24]. In the present study we now show that apoE affects the amyloid fibril formation of synthetic peptides homologous to some other amyloidogenic protein fragments as well. This effect is mediated by the same mechanism, providing further evidence that apoE may indeed, act as a 'universal pathological chaperone'.

Our results indicate that the interaction of various amyloidogenic peptides with apoE is not limited to a specific amino acid sequence, but more related to the physicochemical nature of each fragment, e.g. to properties that are also responsible for the amyloidogenicity. These results are in accordance with those obtained with several mutated and non-mutated A β peptides [34]. The ability of any misfolded amyloidogenic intermediate to assemble into characteristic amyloid fibrils seems to depend on sequence elements that become exposed after partial or complete unfolding. A mutation may directly speed up the fibrillization or just cause a protein to misfold, subsequently leading to the release of the amyloidogenic fragment. In the case of FAF, both mutations causing the disease (Asn and Tyr) lead to premature degradation of gelsolin.

It has been proposed that fragments of only 10 amino acids of AGel (183 FNNGDCFILD 192) [30] and A β (14 HQKLVFFAED 23) [44] would be responsible for the amyloidogenicity and amyloid fibril formation. The most amyloidogenic peptide in PrP is 113 AGAAAAGA 120 [45]. In addition, in several other amyloids only a short sequential stretch is responsible for the amyloidogenicity [46–49]. Thus different amyloid fragments seem to share a relatively short common motif that is responsible for the fibrillogenesis. A conformational change of such a stretch, activated by an external signal (e.g. apoE), seems to be enough to fibrillize the protein. This kind of amyloidogenic consensus sequence has been predicted to exist in various amyloids [50,51]. Observations showing that some originally non-amyloidogenic proteins form amyloid-like structures under suitable conditions support the notion that amyloidogenicity is not limited to only a few protein sequences but rather is a 'hidden' property of many proteins [52–55].

Each amyloidogenic fragment tested in this study competed on the binding to apoE with the other, suggesting that the effect of apoE is mediated through only one common binding site. This is

supported further by the results obtained by quantitative N-terminal sequence analysis of each formed complex, according to which apoE can bind only one amyloid peptide at a time (results not shown). Moreover, several authors have shown *in vivo* and *in vitro* that the binding site for A β is located in a rather restricted area at the C-terminal domain of apoE, further limiting the possibility of several binding sites [22,56,57].

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