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Apolipoprotein E increases the fibrillogenic potential of synthetic peptides derived from Alzheimer's, Gelsolin and AA amyloids

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Abstract **Apolipoprotein E (apoE) has been found in association with several differents types of systemic and cerebral amyloid deposits and the presence of the ε4 allele constitutes a risk factor for Alzheimer's disease. It has been shown that apoE binds and promotes the fibrillogenesis in vitro of Alzheimer's amyloid β-peptide, suggesting an important role for apoE in the modulation of amyloidogenesis. Due to the co-localization of apoE with several biochemically distinct amyloid deposits, it has been proposed that apoE plays a general role modulating and/or participating in amyloidosis. In the present study, we show for the first time that apoE, isolated from human plasma, increases fibril formation of synthetic peptides comprising the amyloidogenic sequences of gelsolin amyloid related to familial amyloidosis Finnish type, and amyloid A found in secondary amyloidosis and familial Mediterranean fever. Our results suggest that apoE acts as a general pathological chaperone in various amyloidoses by enhancing the transition from soluble peptides into amyloid-forming, pathological molecules.**

Key words: Apolipoprotein E; Amyloidogenic conformation; Amyloidosis; Alzheimer's disease; Pathological chaperone

1. Introduction

Amyloidosis is a common feature among many different diseases in which proteins are deposited in the extracellular space of different tissues in the form of amyloid fibers [review 1]. Although amyloid proteins contain different amino acid sequences they all adopt a β-pleated structure in their fibrillar state. Amyloid fibrils are characteristically 8–10 nm wide, unbranched filaments which share common tinctorial properties, including green birefringence after staining with Congo red and binding to the fluorescent dyes thioflavine S and T. Most amyloid proteins show amino- and carboxy-terminal heterogenicity and point mutations have been described in hereditary forms of the amyloid diseases. Although amyloid deposits are believed to be formed by the polymerization of a single protein, certain amyloid associated proteins often are found co-localized in these deposits. Among these amyloid associated proteins, amyloid P-component [2], proteoglycans [3,4], α_1-antichymotrypsin [5], apolipoprotein E (apoE) [6,7] and apolipoprotein J [8,9] appear to be the most abundant. Whether these amyloid associated proteins play an active role in amyloidogenesis or are inert bystanders is at present unclear.

Apolipoprotein E (ApoE) is a 299 amino acid long protein with a molecular weight of 34 kDa, associated with lipid transport [10]. The ApoE gene, located on chromosome 19, has been associated with sporadic and late-onset Alzheimer's disease (AD) [11], a disorder characterized by the massive deposition of a 4.3-kDa amyloid β-peptide (Aβ) in the brain [12,13]. It has been demonstrated that the inheritance of the ε4 allele constitutes a risk factor for AD, modulates the age of onset of the disease and correlates with a greater amount of Aβ deposition in the brain [11,14,15]. The biochemical mechanism to explain the genetic association of apoE4 with AD is, however, unclear. Immunohistochemical studies have shown that apoE is present in a wide variety of systemic and cerebral amyloid deposits [6,7], including secondary amyloidosis and familial Mediterranean fever related to amyloid A (AA), immunoglobulin-related primary amyloidosis, familial amyloidotic polyneuropathy with transthyretin deposition, cystatin C-related hereditary cerebral amyloid angiopathy of Icelandic type, familial amyloidosis of Finnish type (FAF) and spongiform encephalopathies, such as Creutzfeld-Jakob disease, kuru and Gerstmann-Straussler-Scheinker disease.

The wide association of apoE with clinically and biochemically distinct types of amyloidoses has led to the proposal that apoE may have an important general role in amyloidogenesis. This can be the promotion of fibril formation, possibly by inducing β-pleated sheet conformation in amyloidogenic peptides [7]. In this study, we have analysed the effect of apoE on amyloid fibril formation by synthetic peptides containing amyloidogenic sequences of the gelsolin derived amyloid fragment and amyloid A as compared with the effect of apoE on Alzheimer's Aβ peptide.

2. Materials and methods

2.1. Synthetic peptides and proteins

The following synthetic peptides were used (Fig. 1): Aβ1–40 containing the sequence 1–40 of Aβ; Aβ1–40Q bearing the substitution Gln (Q) for Glu (E) at position 22, homologous to the Dutch variant of Aβ [16]; Aβ1–16 used as a control non-fibrillogenic analog of Aβ; $AGel_{wt}$ containing the sequence 183–197 of human gelsolin; $AGel_Y$ containing the same sequence but bearing the substitution of Tyr (Y) for Asp (D) at position 187, found in some cases of FAF; $AGel_N$ containing the substitution Asn (N) for Asp (D) at position 187; Gel165–182 used as a non-fibrillogenic control; AA15 corresponding to the sequence 1–15 and AA14 containing the sequence 2–15 of human AA amyloid, respectively. Aβ and AA peptides were synthesized at the W.M. Keck Facility, University of Yale, by solid phase technique. Aβ1–16 was obtained from Sigma (St. Louis, MO). AGel peptides were purchased from BioSynthesis (Lewisville, TX). All peptides were purified by microbore reverse phase high-performance liquid chromatography (HPLC). The purity of each peptide was evaluated by amino acid composition anal-

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Abbreviations: apoE, apolipoprotein E; AD, Alzheimer's disease; Aβ, amyloid-β peptide; AA, amyloid A; AGel, gelsolin amyloid; FAF, familial amyloidosis of Finnish type; ThT, thioflavine T.

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ysis and laser desorption mass spectrometry. Stock solutions of each peptide were prepared in 50% acetonitrile and the final concentration determined by amino acid composition analysis on a Waters Accu-Tag amino acid analyser.

Human plasma apoE was purchased from Calbiochem (LaJolla, CA). Its purity (>95%) was verified by SDS-PAGE and N-terminal sequence analysis. Stock solutions were made in 0.1 M Tris-HCl, pH 7.4, and quantitated with the Coomassie plus protein assay kit (Pierce).

2.2. Fluorometric assay

Aliquots of peptides were lyophilized, resuspended in 0.1 M Tris-HCl, pH 7.4, and incubated for different times at room temperature. For coincubation experiments, aliquots of apoE to a final molar ratio peptide:apoE of 100:1 were added. Amyloid fibril formation was quantitated by the thioflavine T (ThT) method, as previously described [17]. Briefly, pre-incubated samples were added to 50 mM glycine, pH 9.2, 2 μM ThT to a final volume of 2 ml. Fluorescence was measured at excitation 435 nm and emission 485 nm using a Hitachi F-2000 fluorescence spectrometer. A time scan of fluorescence was performed and three values after the decay reached the plateau (around 300 s) were averaged after substracting the background fluorescence of 2 μM ThT. All the experiments were performed in triplicate.

2.3. Electron microscopy

Peptides (1 mg/ml) were incubated with or without apoE in 0.1 M Tris-HCl, pH 7.4, for 5 days at room temperature. Aliquots of each sample were then spotted on a carbon formvar-coated 400-mesh nickel grids for 1 min, washed with distilled water and stained for 90 s with 2% uranyl acetate. Grids were visualized on a Zeiss EM 10 electron microscope at 80 kV.

2.4. Sedimentation assay

Peptides were incubated in 0.1 M Tris-HCl, pH 7.4, for different times at room temperature. To separate the soluble and aggregated peptide, each sample was centrifuged at 14,000 rpm for 15 min in an Eppendorf microfuge. Aliquots of the supernatant were analysed by microbore HPLC using a reverse phase Vydac C4 column (214 TP 52) and a linear gradient of 3–70% acetonitrile in 0.1% TFA in 25 min. Absorbance was monitored at 220 nm. The percentage of the non-sedimentable peptide was measured by comparing the area of the peak corresponding to the soluble peptide at diferent times of incubation with that obtained with the non-incubated sample.

3. Results and discussion

All synthetic peptides modeled on the amyloidogenic sequence of Aβ (Fig. 1A), AGel (Fig. 1B) and AA (Fig. 1B) were able to produce amyloid-like fibrils with morphological characteristics typical of amyloid (Fig. 2). The fluorometric assay was used to measure the effect of apoE on amyloid fibril formation by these synthetic peptides. At a molar ratio of 1:100 (apoE:peptide), human plasma apoE clearly promotes amyloid formation of all the amyloidogenic peptides, as shown in Table 1. This molar ratio was used in order to mimic the conditions locally occuring in amyloid, considering the yield of apoE fragments extracted from brain and systemic amyloid deposits [18,19]. The effect of apoE was maximal up to 24 h after which only a small amount of additional amyloid could be detected. Interestingly, the promoting effect of apoE was higher in those peptides which were less amyloidogenic ($AGel_Y$, Aβ1–40 and AA14) and lower in those peptides that were more amyloidogenic ($AGel_N$, Aβ1–40Q and AA15) (Table 1). These results suggest that apoE promotes amyloidogenesis by enhancing the fibrillogenic potential of peptides less able to form amyloid. Electron microscopic examination of the incubated samples showed that morphologically similar amyloid-like fibrils were formed by the amyloidogenic peptides in the presence or absence of apoE (data not shown).

In order to quantitate the level of peptide aggregation, we measured the amount of remaining soluble peptide after different times of incubation by the sedimentation assay (Fig. 3). Each peptide aggregated only partially in the absence of apoE, even after long incubation times (>120 h) (Fig. 3). The exception was AA15 which sedimented almost completely after

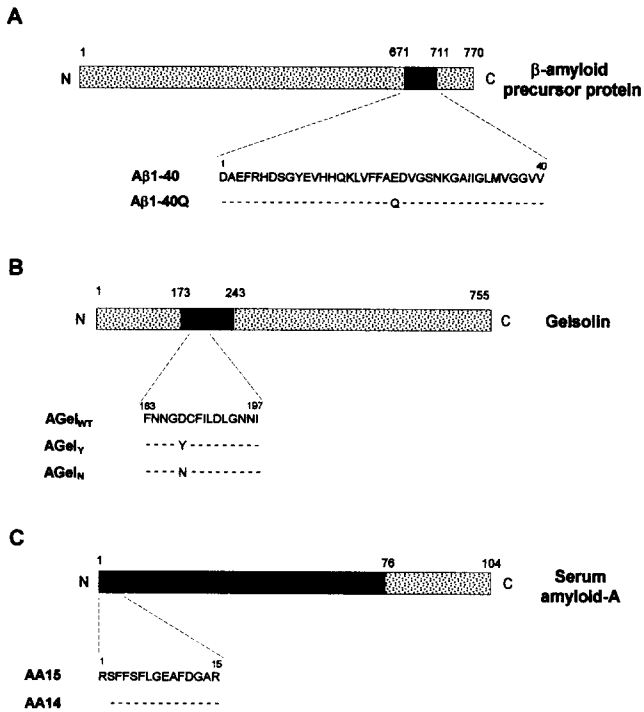

Fig. 1. Schematic representation of the amyloid precursor proteins, the fragments deposited as amyloid in the tissues and the synthetic peptides used as a model in the present study. (A) Alzheimer's β-amyloid, (B) Gelsolin amyloid and (C) AA amyloid. The black box represents the peptide fragment most commonly deposited as amyloid in the tissues. In the case of the Alzheimer's β-amyloid precursor protein and serum amyloid A protein, only one isoform is represented.

Table 1
Effect of human plasma apoE on amyloid formation by Agel, AA and Aβ analogs

Peptide	ThT-fluorescence		apoE[b] enhancement
	Peptide alone	Peptide with apoE	
AGel183–197^{WT}	3.6 ± 0.2	7.7 ± 0.4	2.14
AGel183–197^{Y}	1.6 ± 0.3	13.9 ± 2.3	8.69
AGel183–197^{N}	17.6 ± 1.9	22.2 ± 2.2	1.26
Gel165–182	0.53 ± 0.1	0.58 ± 0.05	1.09
AA14	2.9 ± 0.6	16.9 ± 3.7	5.83
AA15	14.1 ± 0.7	14.3 ± 1.5	1.01
Aβ1–40[a]	15.2 ± 1.1	33.8 ± 2.9	2.22
Aβ1–40Q[a]	33.8 ± 0.4	38.1 ± 2.5	1.13
Aβ1–16	0.39 ± 0.04	0.34 ± 0.07	0.87

30-μg aliquots of the peptides were incubated with or without apoE in a molar ratio 1:100 in 30 μl of 0.1 M Tris, pH 7.4, for 24 h. Amyloid formation was quantitated by ThT-fluorescence in triplicate. ApoE alone did not produce any fluorescence above the background.
[a]The effect of apoE on the amyloid formation by Aβ1–40 and Aβ1–40Q was already published as part of a previous paper [28].
[b]ApoE enhancement correspond to the increment of amyloid formed by the peptides incubated with apoE as was calculated by dividing the fluorescence values obtained in the presence and in the absence of apoE.

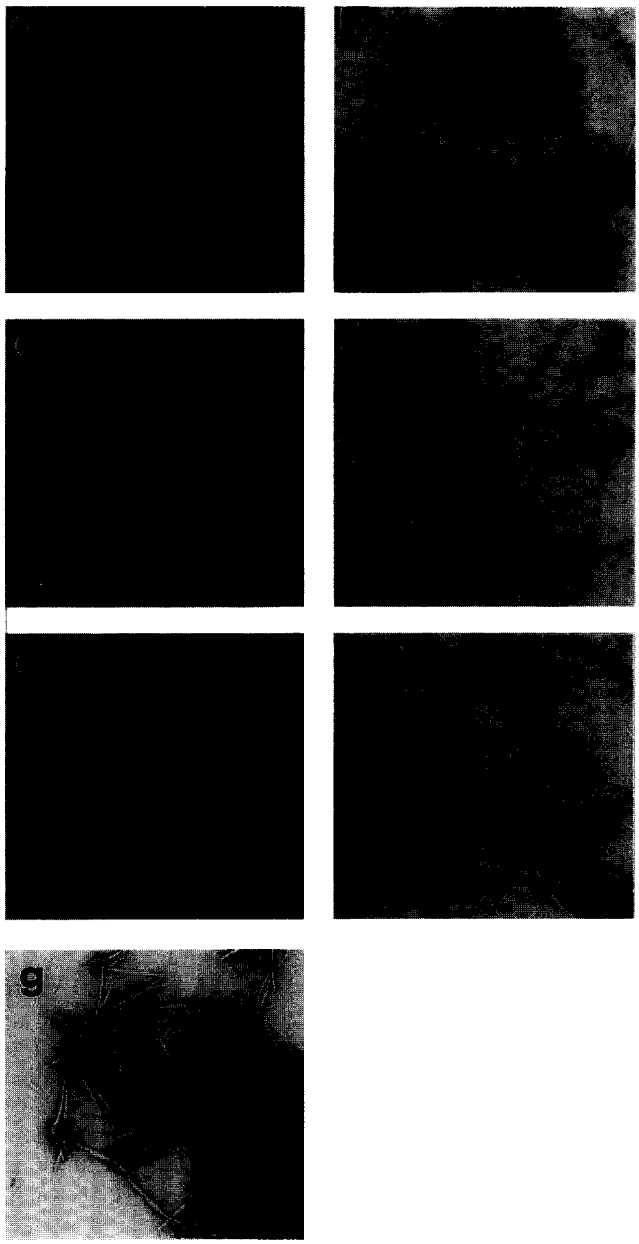

Fig. 2. Electron micrographs of negatively stained amyloid-like fibrils produced in vitro by synthetic peptides containing the amyloidogenic sequence of Aβ, AGel and AA. Aliquots of peptides at 1 mg/ml were incubated for 5 days at room temperature and the fibrils were visualized as described in Section 2. (a) Aβ1–40; (b) Aβ1–40Q; (c) AA14; (d) AA15; (e) $Agel_{wt}$; (f) $AGel_{y}$; (g) AGelN. The peptides used as controls (Aβ1–16 and Gel165–182) did not produce amyloid-like fibrils. The scale bar represents 100 nm.

5 days of incubation. Addition of apoE to the remaining supernatant of $AGel_{wt}$, Aβ1–40 and AA14 after 6 days of incubation, reinitiated precipitation (Fig. 3). The control peptides (Aβ1–16 and Gel165–182) remained 100% soluble after times longer than 6 days and were not affected by apoE. One explanation of our findings is that apoE decreases the critical concentration of aggregation, i.e., the minimum concentration essential for peptide interaction, by favoring the intermonomeric association to give rise to amyloid. Alternatively the results can be due to the transformation by apoE of some soluble non-amyloidogenic peptides to a state capable of ordered aggregation. If the fibrillogenesis promotion by apoE is due to the induction of a decrease in the critical concentration for aggregation, this effect should be mimicked by concentration of remaining soluble sample.

In order to test these two alternatives, we concentrated the peptide fraction remaining soluble after 6 days of incubation of $AGel_{wt}$, AA14 and Aβ1–40 to 2 mg/ml and continued the incubation. Each peptide remained soluble even after several days of incubation in the absence of apoE, but they rapidly aggregated in the presence of apoE (data not shown). These results suggest that apoE promotes fibrillogenesis by changing the conformation of soluble peptides, incompetent to form amyloid fibrils, to a state which they rapidly form amyloid like aggregates.

Synthetic peptides homologous to proteins or peptides fragments isolated from amyloid deposits, have been extensively used as a model to study fibril formation in vitro. It has been shown that the complete sequence 1–40 or 1–42 of Aβ (Fig. 1A) and also shorter derivatives thereof can form amyloid-like fibrils in vitro exhibiting a β-pleated sheet conformation, similar to that found in Alzheimer's amyloid plaques (for a review, see [20]). Similarly, synthetic peptides containing a part of the sequence of gelsolin derived amyloid fragment with the substitutions Asp to Asn or Tyr at position 187 form ultrastructurally amyloid-like fibrils in vitro [21]. The shortest gelsolin peptide capable of forming amyloid-like fibrils was reported to be a 9 amino acid long fragment corresponding to the sequence 183–191 [21]. In secondary or reactive amyloidosis, protein AA fragments have been isolated from amyloid deposits associated with chronic infections, inflamatory disorders and certain neoplasms [22,23]. AA amyloid corresponds to the N-terminal fragment formed by cleavage and removal of the C-terminal part of a larger precursor protein, called serum amyloid A (Fig. 1C). It has been shown that several peptides containing a part of the N-terminal fragment of serum amyloid A can form amyloid-like fibrils in vitro [24]. The shortest fragment reported to form amyloid-like fibrils corresponds to a 13 residue long peptide with the sequence 2–12 of AA [24].

ApoE is a ubiquitous protein, associated with several types of cerebral and systemic amyloidoses [6,7]. It has been proposed that apoE acts as a pathological molecular chaperone in disorders in which amyloid material is accumulated. Pathological chaperones are defined as proteins mediating β-pleated amyloid formation of polypeptide fragments [7]. Recently, it has been shown that apoE binds to Aβ [14,25], promotes Aβ amyloid formation in vitro [26–28] and induces conformational changes in Aβ to give rise to the amyloidogenic β-sheet conformation of this peptide (Soto et al., in prep.). Our present findings show for the first time that apoE can also enhance the fibrillogenesis of other amyloid-related peptides, suggesting that the effect of apoE is not specific for amyloid formation by Aβ, but rather reflects the general ability of apoE to stabilize or induce β-sheet structure in peptides bearing the potential to adopt this conformation. Our data indicate that apoE has a general role in amyloidogenesis by acting as a universal pathological chaperone, binding hydrophobically to a common conformation shared by different amyloid peptides, regardless of their amino acid sequence. Recently, it has been shown that the carboxyl-terminal domain of apoE copurifies with AD senile plaques [18] and with amyloid deposits derived from amy-

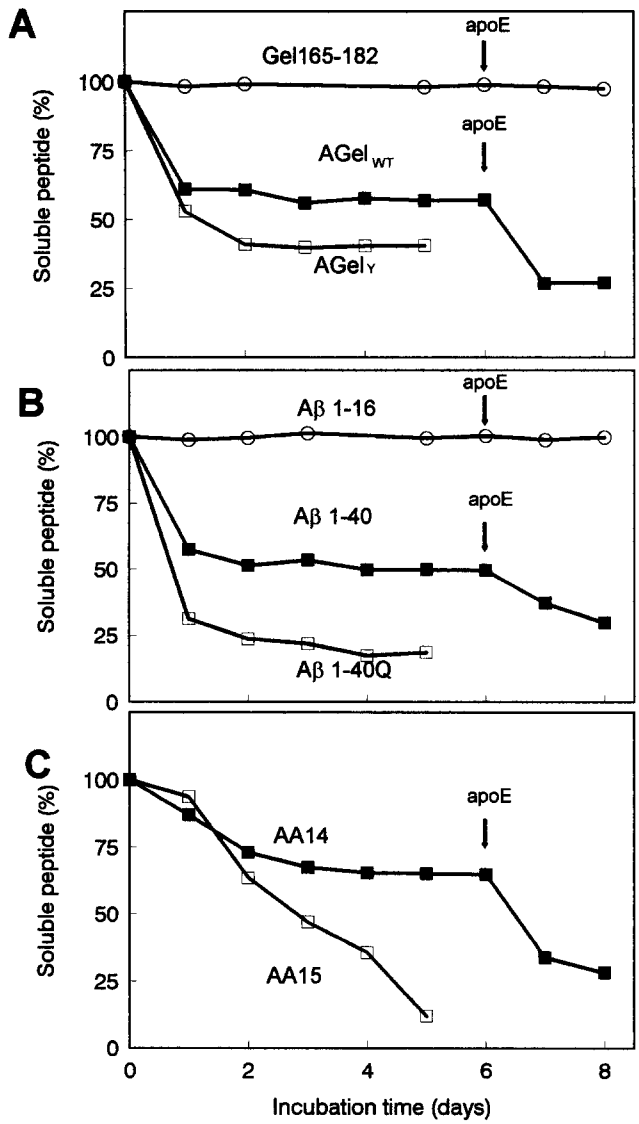

Fig. 3. Percentage of soluble peptide after different incubation times and effect of apoE. 100 μg of synthetic peptides containing the amyloidogenic sequence of gelsolin (A), Aβ (B) and AA (C) amyloids were incubated in 50 μl of 0.1 M Tris, pH 7.4, at room temperature. At the indicated times, the samples were centrifuged at 14,000 rpm for 15 min and 3 μl of the supernatant was subjected to reverse phase HPLC. The percentage of the soluble peptide was determined as described in Section 2. Arrow indicates when an appropiate amount of apoE to get a final molar ratio (apoE:peptide) 1:100, was added to the solution.

loid A and amyloid L [19]. Moreover, the C-terminal fragment of apoE can form amyloid-like fibrils in vitro [18], leading to the idea that, when an amyloid-associated protein is misfolded, it can propagate this misfolding by acting as a template for another protein that does not share amino acid sequence homology [29]. This conformational mimicry may explain the effect of apoE upon various sequentially unidentical amyloid peptides.

In this study we used pooled apoE isolated from human plasma, which contains a mixture of the three isoforms, but according to the statistical distribution of apoE isoforms in the population, corresponds mainly to the isoform apoE3. The human plasma apoE was used since it has been reported that there is no association between e alleles and systemic amyloid disorders and that recombinant apoE isoforms are less active than native pooled apoE [28]. The genetical association of apoE4 to some amyloid diseases might be a consequence of a more complicated cascade of events rather than a direct protein–protein interaction. Alternatively, it is possible than certain apoE isoforms are more efficient promoters of amyloid formation by some amyloidogenic peptides than others, as apoE4 appears to be the most effective promotor of Aβ fibrillogenesis in vitro [26,27].

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