Apolipoprotein E structural requirements for the formation of SDS-stable complexes with β-amyloid-(1–40): the role of salt bridges

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Of the three major isoforms of human apolipoprotein E (apoE), apoE4 is a risk factor for the development of Alzheimer's disease. Among possible neurologically relevant differences in the properties of apoE3 and apoE4 is the fact that apoE3 forms an SDS-stable complex with β-amyloid-(1–40) (Aβ40) with greater avidity than does apoE4. This interaction may sequester potentially toxic species of Aβ or facilitate clearance. To understand more about this difference, we examined whether differences in salt bridges between apoE domains influence the capacity of apoE isoforms to form complexes with Aβ. In apoE3 there is a salt bridge between Arg-61 and Asp-65, while in apoE4 there are salt bridges between Arg-61 and Glu-255, and Arg-112 and Glu-109. Mutation of position 112, which is Cys in apoE3 and Arg in apoE4, to Ala or Lys abolished complex formation, while mutant apoE with Ser at this position retained the capacity to form complex. Substituting Ala for Glu-109 had no effect on the ability of either apoE4 or apoE3 to form complexes. On the other hand, substitution of Thr for Arg-61 in apoE3 abolished, and truncation of apoE3 at position 201 substantially lowered, but did not abolish, complex formation. Neither of these mutations within apoE4 had any affect on its complex formation with Aβ. These results suggest that the nature of the cysteine residue in apoE3 and interactions between the N-terminal and C-terminal domains of human apoE are important for the ability of apoE3 to form an SDS-stable complex with Aβ40.

Key words: Alzheimer's disease, apoE3/apoE4 isoforms, site-directed mutants.

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease affecting increasing numbers of elderly individuals. One of the hallmarks of this disease is the deposition of amyloid plaques, mainly in the hippocampus and neocortex [1]. These plaques include β-amyloid (Aβ) peptides that contain between 39 and 43 amino acids, which are produced by processing of the ubiquitously expressed amyloid precursor protein (APP) [2]. The importance of this pathway from APP to Aβ is demonstrated by the increased incidence of AD, cerebral amyloidosis and plaque formation in humans and transgenic animals carrying mutants of APP that increase the production of Aβ [2–5]. Genetic mutations in presenilin genes also influence APP processing, resulting in an increase in the Aβ-(1–42)/Aβ-(1–40) (Aβ40) ratio [6,7], and an increased incidence of AD in humans [2] and of amyloid deposition in transgenic mice containing mutants of presenilins and Aβ [8].

Apolipoprotein E (apoE) is a 35 kDa protein comprising 299 amino acids that is expressed in the human population in one of three major isoforms [9]. ApoE2 (Cys-112/Cys-158), apoE3 (Cys-112/Arg-158) and apoE4 (Arg-112/Arg-158) differ from each another by amino acid changes at positions 112 and 158. Thus apoE3 and apoE4 differ by Cys/Arg exchange at position 112. ApoE is produced in multiple cell types and tissues, with the liver and brain being the tissues with the highest level of apoE expression. The apoE produced by the liver is associated with lipoproteins, predominantly very-low-density lipoprotein (VLDL) and high-density lipoprotein (HDL). In the plasma, apoE4 preferentially associates with VLDL, while apoE3 is mostly found in HDL [10,11]. In the brain, apoE is secreted by astrocytes and microglial cells [12]. The apoE secreted by astrocytes is in HDL-size particles [13,14]. In humans, apoE is also present in neurons [15].

It is now well established that apoE4 is a risk factor for the development of sporadic and familial late-onset AD [16]. The presence of apoE4 results in a greater amyloid burden [17] and an age-related increase in Aβ accumulation [18]. A number of studies have attempted to identify differences in the neurologically relevant functions of apoE4 and apoE3 that might account for the association of apoE4 with AD. These include their differential ability to form SDS-stable complexes with Aβ [19–21], to function as chaperones in aggregation of Aβ [22,23], to function as antioxidants [24], to modify the cytotoxicity of Aβ towards neuronal cells [25,26], to respond to focal ischaemia [27] or neurodegeneration [28], to promote neurite outgrowth in cultured neuronal cells [29,30] and to influence the deposition of Aβ in transgenic mice expressing APP mutants [31,32], as well as differences in the precise nature of the lipoproteins secreted by cultured astrocytes expressing either human apoE3 or apoE4 [33].

ApoE contains two major domains [34,35]. The N-terminal domain (residues 1–191) contains the residues that confer isoform-specificity (residues 112 and 158) and the receptor binding domain (residues 130–150). Structural studies indicate that the lipid-free form of the N-terminal domain assumes a four-helix bundle [9]. The C-terminal domain (residues 216–299) is α-helical and binds lipids. ApoE3 and apoE4 differ in conformation as a result of differences in interactions between the N- and C-terminal domains that are determined, at least in part, by differences in salt bridges [9]. In apoE3, Asp-65 forms a salt bridge with Arg-61. On the other hand, the presence of Arg-112

in apoE4 results in a salt bridge forming between Glu-109 and Arg-112, a re-orientation of Arg-61, and the formation of a new salt bridge between Arg-61 and Glu-255 in the C-terminus of apoE [9,36], thus linking the N-terminus and C-terminus of the protein. These structural differences are thought to account for the preferential association of apoE isoforms with distinct classes of lipoproteins [36,37]. Whether the salt-bridge-mediated conformational differences between apoE3 and apoE4 account for differences in the neural effects of these isoforms is not clear.

As mentioned above, apoE3 forms an SDS-stable complex with $A\beta$ with greater avidity than does apoE4 [19–21]. We have postulated that such complex formation may facilitate the detoxification and/or clearance of $A\beta$ [25,38]. In the present study, we investigated whether differences between the salt bridges in apoE3 and apoE4 could account for the preferential association of apoE3 with $A\beta$. The rationale for this study is based on results from work by Weisgraber and colleagues [11,36,37] on apoE isoform-specific associations with lipoproteins, where it was found that apoE4 exhibits a twofold preference for VLDL, which appears to be attributable, at least in part, to salt bridges between the N-terminus and C-terminus of apoE.

EXPERIMENTAL

Mutagenesis of human apoE cDNA

Human apoE3 and apoE4 cDNAs [19] were subcloned into the *Kpn*I and *Xba*I sites of M13mp18. Single-stranded M13 DNAs were used as templates for site-directed mutagenesis (Bio-Rad T7 *in vitro* mutagenesis). Mutagenesis was confirmed by DNA sequencing of the single-stranded DNA. The double-stranded DNAs were subcloned into the *Kpn*I and *Xba*I sites of pCMV4.

Expression of wild-type and mutant human apoEs in cultured cells

The human apoE mutants were expressed in HEK-293 cells (A.T.C.C. CRL1573) by transient transfection or as stably transfected cells as described for wild-type apoE [19], except that LIPOFECTAMINE™ transfection reagent (Life Technologies) was used to transfect the cells with pCMV4 vectors containing the apoE mutants, and pCMV/zeo plasmid (Invitrogen) was used instead of pSV40Neo to generate stably transfected cells. Stably transfected cells were selected in 30 μg/ml Zeocin. Serum-free medium was added to both stably and transiently transfected cells for 3 days, the medium was then concentrated $\sim$ 50–70-fold and the amount of apoE in the medium was quantified by densitometry of SDS/polyacrylamide gels using serial dilutions of the medium and apoE standards of known concentration. Medium from mock-transfected cells was concentrated to the same extent. ApoE represents $\sim$ 50–80 % of the Coomassie Blue-stainable protein in the medium, with the other stainable proteins being residual albumin from the culture medium and the protease inhibitor aprotinin [19]. These latter proteins are also present in mock-conditioned media and do not form complexes with $A\beta$. Since apoE does not have a high affinity for Coomassie Blue, this method probably underestimates the amount of apoE in the medium. This is borne out by the fact that more than 80 % of the radiolabelled protein secreted by transfected HEK cells is apoE [19].

ApoE–$A\beta$ complex formation and detection

Complex formation and detection were performed following the procedure outlined in LaDu et al. [19]. Briefly, conditioned medium containing 0.5 μg of apoE (final concentration 25 μg/ml)

or an equal volume of mock-conditioned medium was incubated at room temperature for 2 h with 250 μM $A\beta$40 (California Peptide Research Inc.) in PBS (pH 7.4) in a total volume of 20 μl. The reaction was stopped by the addition of 20 μl of 2 × non-reducing Laemmli buffer [19]. Complexes in 10 μl of the binding assay were separated on 10–20 % polyacrylamide SDS/Tricine gels (Novagen) in the absence of 2-mercaptoethanol, transferred to an Immobilon-P membrane (Millipore), and probed with 4G8 monoclonal antibody to $A\beta$ (Senetek; 1:3000 dilution), a polyclonal rabbit antibody to human apoE (1:5000 dilution) produced by immunization of rabbits with purified human apoE, or the 1D7 monoclonal antibody to apoE (Ottawa Heart Institute; 1:1000 dilution). ApoE proteins on Western blots were visualized by enhanced chemiluminescence (Amersham) and the film was quantified by densitometry using Advance Quantifier (BioImage) to confirm that approximately equivalent amounts of apoE protein were added to each of the binding reactions. In most cases the relative levels of apoE–$A\beta$ complexes were not determined, because, in order to observe lower-affinity complexes, it was necessary to overexpose the apoE3–$A\beta$ complex.

RESULTS

Wild-type apoE isoforms

In all experiments the ability of the mutants to associate with $A\beta$40 is compared with that of wild-type apoE3 or apoE4. All blots were probed with antibodies to $A\beta$ to detect SDS-stable complexes, and with antibodies to apoE to confirm that equivalent amounts of apoE were used in each assay. As we have previously shown [19], apoE3 readily forms an SDS-stable complex with $A\beta$, while apoE4 binds much less avidly (see Figures 2–5). ApoE3 dimers are also detected on these non-reducing gels. There is a protease-sensitive site in apoE between residues 191 and 216 which results in cleavage of apoE into 22 kDa and 12 kDa peptides [9]. ApoE4 appears to be more sensitive to proteolysis than apoE3. The 12 kDa C-terminal fragment (which is more readily detected by the polyclonal antibody than the 22 kDa N-terminal fragment) represents less than 10 % of total apoE4 protein. This difference in protease sensitivity may indicate differences in the conformation of the two proteins.

Importance of Cys-112

The apoE3 and apoE4 isoforms differ from each other due to a cysteine–arginine interchange at position 112. In the crystal structures of the lipid-free N-terminal domains of the two isoforms, this amino acid exchange results in changes in several salt bridges within the molecule [9]. In apoE3, there is a salt bridge between Arg-61 and Asp-65 (Figure 1). In apoE4, Arg-112 forms a salt bridge with Glu-109 and, in so doing, modifies the position of the side chain of Arg-61. In its modified position, Arg-61 appears to interact with Glu-255 in the C-terminus, possibly through a salt bridge [36]. The question arises as to whether Cys-112 is a positive contributor to the interaction of apoE3 with $A\beta$, or whether the presence of Arg-112 abrogates this interaction by promoting the salt bridge with Glu-109. A related issue is whether arginine is specifically required for formation of a salt bridge with Glu-109, or whether lysine would function similarly. To examine this question, the residue at position 112 was substituted with alanine, a neutral amino acid, or lysine, a positively charged amino acid. A mutant containing lysine rather than arginine at position 112 (apoE-Lys-112), which could potentially maintain the salt bridge with Glu-109, was as ineffective as apoE4 in forming an SDS-stable complex with $A\beta$ (Figure 2a). This result
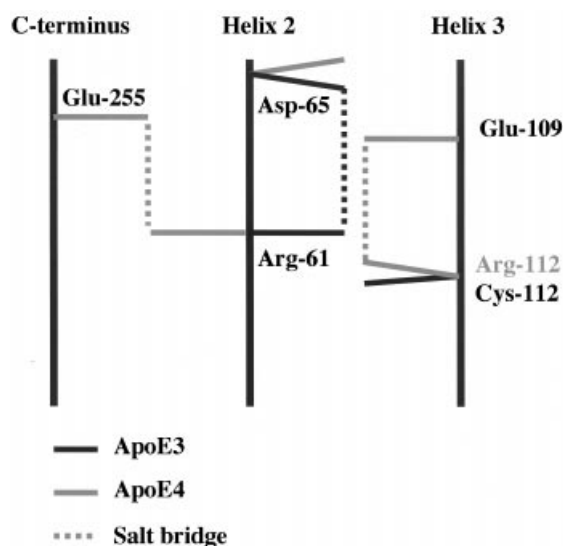
Figure 1   Representation of known and putative salt bridges that differ between apoE3 and apoE4

The amino acid side chains (horizontal solid bars) and salt bridges (broken lines) in helices 2 and 3 of the crystal structure [9] and the C-terminal domain are shown. Features of apoE3 are shown in black, and those of apoE4 in grey. In apoE3, Arg-61 and Asp-65 form a salt bridge. The substitution of Cys-112 for Arg in apoE4 results in a new salt bridge forming between Arg-112 and Glu-109. This is thought to change the orientation of Arg-61 so that it now forms a salt bridge with Glu-255 instead of Asp-65.

Figure 2   Effects of substitutions at position 112 in apoE

Shown are Western blots (representative of three experiments) of binding reactions containing 25 μg/ml apoE3, apoE4, apoE-Ala-112, apoE-Lys-112 (**a** and **b**) or apoE-Ser-112 (c) or media from mock-transfected cells, incubated with or without 250 μM Aβ40 peptide for 2 h at room temperature, as described in the Experimental section. The samples were run on SDS/Tricine gels, transferred to Immobilon-P membranes and probed with 4G8 antibody to Aβ (**a** and **c**) or anti-apoE serum (**b**). Each lane is identified by the nature of the apoE protein and an indication of whether Aβ40 was absent (−) or present (+) in the binding assay. Molecular mass markers (kDa) are shown on the right.
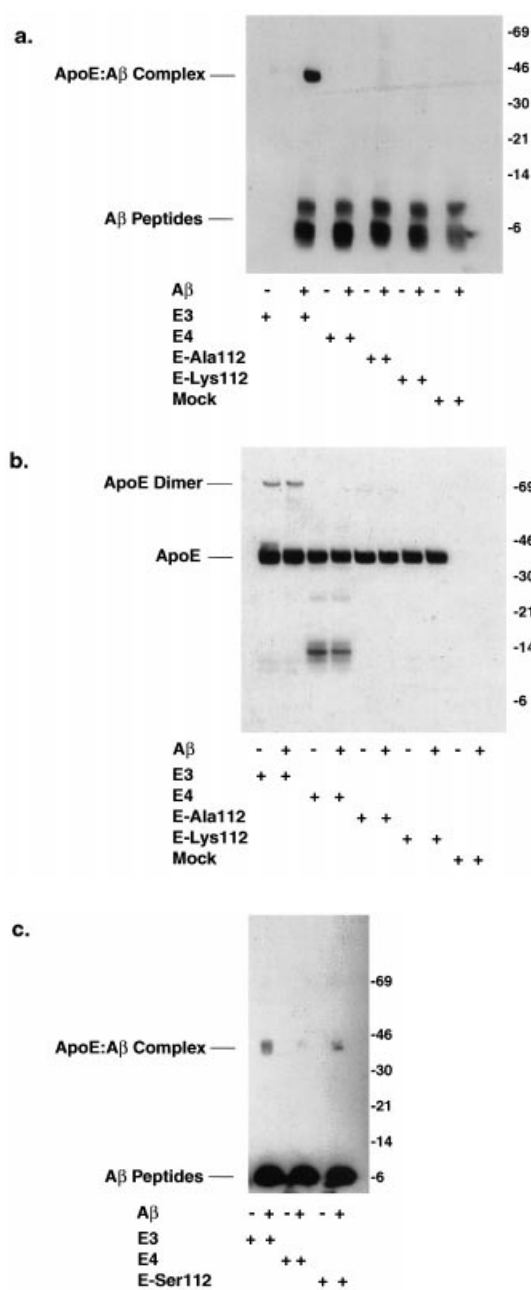
was not unexpected. On the other hand, replacing Cys-112 with alanine (apoE-Ala-112), which should have little or no influence on the salt bridges within the apoE3 isoform, resulted in a protein that was also as poor as apoE4 at forming an SDS-stable complex with Aβ (Figure 2a). This argues that the cysteine residue at position 112 may be very important, if not critical, for the stable interaction of apoE3 with Aβ. To confirm whether cysteine is specifically required at position 112, we replaced it with serine, a structurally similar amino acid. As shown in Figure 2(c), apoE-Ser-112 was able to form a stable complex with Aβ, albeit ∼ 20 % less effectively than wild-type apoE3.

Elimination of the Arg-112–Glu-109 salt bridge does not change the relative abilities of apoE3 and apoE4 to form an SDS-stable complex with Aβ

As a further test of the role of the Arg-112–Glu-109 salt bridge in apoE4, Glu-109 was substituted by an alanine residue in both apoE3 and apoE4. As shown in Figure 3, this change had relatively little impact on the ability of apoE3 and apoE4 to form complexes with Aβ, since apoE3 and apoE3-Ala-109 formed similar amounts of complex, as did apoE4 and apoE4-Ala-109. ApoE3-Ala-109 retains the capacity to form dimer. This is consistent with the Ala-112 substitution, and suggests that the formation of an Arg-112–Glu-109 salt bridge in apoE4 does not impact on complex formation with Aβ.

### Arg-61 is critical for apoE3 to form a complex with Aβ

Human apoE is unique among the known sequences of apoE from different species in having an arginine residue at position 61. Since in most other species position 61 is threonine, we substituted threonine for arginine at position 61 in both apoE3 and apoE4. This mutation was designed to disrupt the salt bridge

in apoE3 between Arg-61 and Asp-65 and that in apoE4 between Arg-61 and Glu-255. Neither apoE3-Thr-61 nor apoE4-Thr-61 was able to complex with Aβ under our assay conditions (Figure 4). The substitution of threonine for arginine in apoE3 completely abolished its ability to complex with Aβ, but not its ability to form dimer. There may have been a very modest
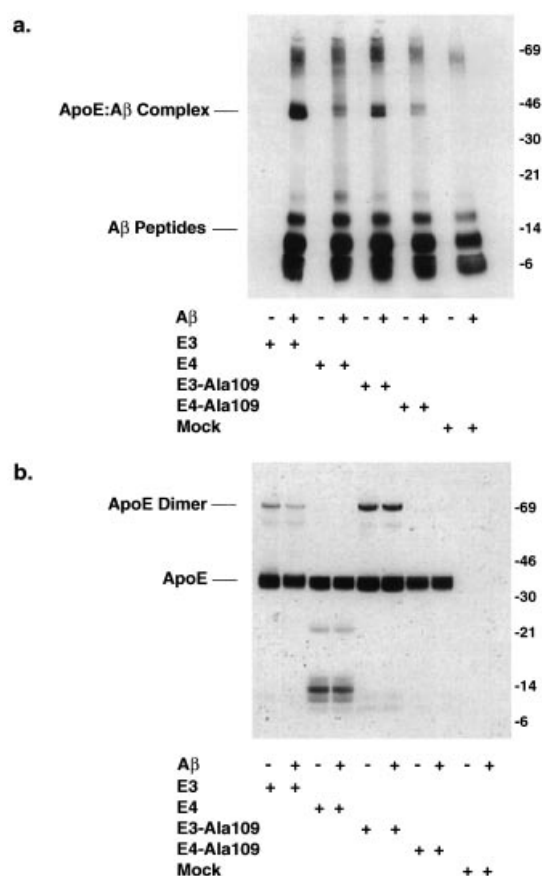
Figure 3   Effects of substitutions at position 109 in the apoE isoforms

Shown are Western blots (representative of two experiments) of binding reactions containing apoE3, apoE4, apoE3-Ala-109, apoE4-Ala-109 or mock medium incubated in the presence or absence of Aβ as described in the legend to Figure 2. The blots were probed with 4G8 antibody (**a**) and anti-apoE serum (**b**).
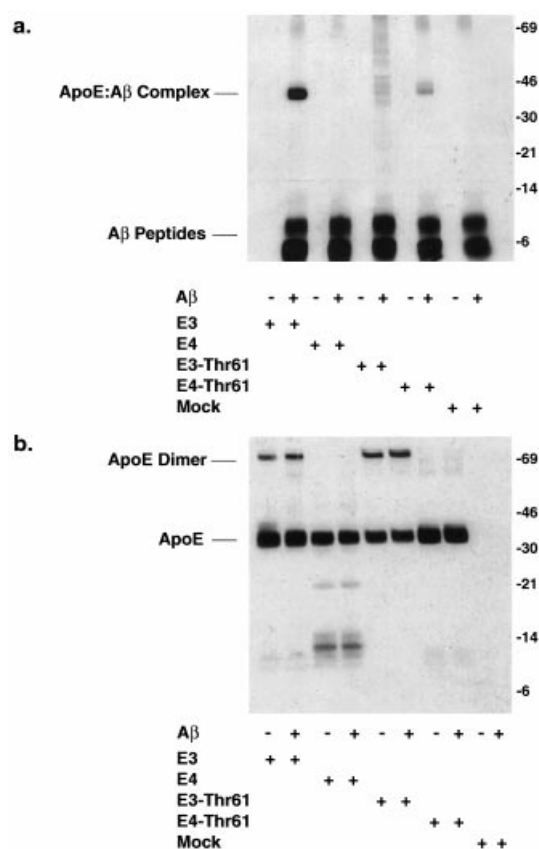

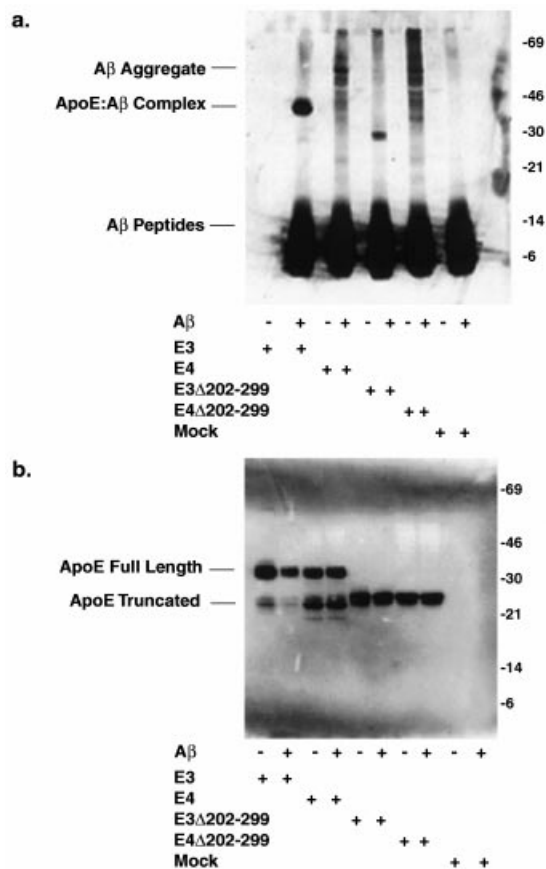**Figure 4   Effects of substitutions at position 61 in the apoE isoforms**

Shown are Western blots (representative of two experiments) of binding reactions containing apoE3, apoE4, apoE3-Thr-61, apoE4-Thr-61 or mock medium incubated in the presence or absence of Aβ as described in the legend to Figure 2. The blots were probed with 4G8 antibody (**a**) and anti-apoE serum (**b**).

enhancement of the ability of apoE4-Thr-61 to form complexes, although this is probably attributable to the fact that ∼ 40 % more of this apoE mutant was used in the assay (Figure 4b).

The N-terminal fragment of apoE3 retains a limited capacity to form a complex with Aβ

ApoE has two major domains – an N-terminal structural domain (residues 1–191) which, in the absence of lipid, assumes a four-helix bundle, and a C-terminal lipid binding domain (residues 216–299) [34,35]. These are linked by a hinge domain spanning residues 191–215. It has previously been suggested that the lipid binding domain is important for the interaction of apoE3 with Aβ [39,40]. One possible explanation for the inability of apoE3-Thr-61 to form complex might be that the interaction between the N- and C-terminal domains may be important for complex formation. To explore the influence of the C-terminal domain, we truncated both apoE3 and apoE4 at residue 201. The results are illustrated in Figure 5. Because the polyclonal antibody used to detect apoE was not equally reactive with full-length and truncated apoE, we used the monoclonal antibody 1D7 (against residues 140–160) to detect apoE in these blots, as this antibody was equally reactive with the wild-type and truncated apoE. Complex formation with apoE3Δ202–299 could only be observed with prolonged exposure of the Western blot (Figure 5a), but

not with standard exposure. Even under these conditions virtually no complex was seen with either apoE4 or apoE4Δ202–299. Thus, while the N-terminal domain can bind Aβ, the optimal interaction appears to involve both the N-terminal and C-terminal domains.

This overexposed blot revealed another interesting property of apoE4: both the full-length and truncated apoE4 appeared to catalyse the formation of Aβ aggregates. Very little Aβ aggregation was seen with apoE3 or apoE3Δ202–299. Furthermore, the Aβ aggregates did not contain significant quantities of apoE (Figure 5b). This indicates (as has been reported by others [22,23]) that, despite the inability of apoE4 to form a stable complex with Aβ, it may facilitate Aβ aggregation in a manner that does not involve the SDS-stable association of apoE4 with Aβ.

DISCUSSION

We investigated the effects of salt bridge formation between the N- and C-termini of apoE on isoform-specific interactions with Aβ40. If the domain interaction was important in accounting for the markedly decreased capacity of apoE4 to form an SDS-stable complex with Aβ, then disruption of the salt bridge in apoE4 between residues 112 and 109, with a resultant change in the orientation of Arg-61 and the formation of a salt bridge

Figure 5   Effects of truncation of the apoE isoforms at residue 201

Shown are Western blots (representative of four experiments) of binding reactions containing apoE3, apoE4, apoE3Δ202–299, apoE4Δ202–299 or mock medium incubated in the presence or absence of Aβ as described in the legend to Figure 2. The blots were probed with 4G8 antibody (**a**) and 1D7 antibody to apoE (**b**). The Western blot in (**a**) was overexposed in order to detect the small amount of complex formed with the truncated apoE mutants. The monoclonal antibody was used since it recognized both the full-length and truncated apoE equally, unlike the polyclonal antibody.

between Arg-61 and Glu-255, should enhance complex formation. However, mutation of Glu-109 to alanine in apoE4 did not enhance complex formation, and the same mutation in apoE3 had little effect. In addition, conversion of Arg-61 into threonine, a residue found in this position in most other species of apoE, also did not significantly enhance apoE4 complex formation. Finally, the removal of the C-terminal domain (residues 202–299) substantially reduced the capacity of even apoE3 to form complexes, indicating the importance of this region of the protein, regardless of salt bridge differences between apoE3 and apoE4. Even when domain interactions were abrogated due to the absence of the C-terminal fragment, the small amount of residual complex formation between the apoE3 N-terminal fragment and Aβ was still greater than the amount of complex formation with the apoE4 fragment. This result is consistent with that of Golabek et al. [41], who demonstrated binding of Aβ40 to both the N-terminal and C-terminal domains of apoE3, with higher-affinity binding to the N-terminal domain. From these results, we conclude that the preference of apoE3 over apoE4 for SDS-stable complex formation with Aβ40 is not simply attributable to domain interactions mediated by salt bridges between residues 109 and 112 on the one hand, and residues 61 and 255 on the other.

The cysteine residue, or at least a hydrophilic residue lacking a positive charge, seems to be very important for complex formation with Aβ. ApoE3, apoE2 and rabbit apoE all contain a cysteine at position 112 and are competent to form complexes with Aβ [42]. Serine, but not arginine, can substitute for cysteine in this position and maintain complex formation with Aβ. A mutant containing alanine at position 112 does not retain this ability. It is possible that this alanine disrupts the amphipathic character of helix 3 and so prevents its interaction with Aβ. Whether it is the hydrophilicity of the cysteine residue or its space-occupying character that is important in the formation of complex with Aβ is not clear. It is possible that the Arg-61–Asp-65 salt bridge of apoE3 is important for its ability to complex with Aβ, since a mutation of Arg-61 to threonine, which would disrupt this salt bridge, also largely eliminates the capacity for complex formation. It is thought that this salt bridge is also disrupted in apoE4. Mutation of Glu-109 to alanine has little effect on complex formation by apoE3, and this is not unexpected. However, this mutation in the apoE4 context does not increase the ability of apoE4 to complex with Aβ, as might have been expected if it helped to sustain an all important Arg-61–Asp-65 salt bridge. This suggests that the salt bridge between Glu-109 and Arg-112 is not responsible for the disruption of the Arg-61–Asp-65 salt bridge, or that some feature other than disruption of this salt bridge is crucial in relation to the relative inability of apoE4 to form complexes. This argument re-inforces the critical positive influence of the cysteine (or serine) residue in apoE3 with regard to its ability to complex with Aβ.

While the present study involved primarily single-site mutations of apoE3 and apoE4, the conclusions have to be tempered by the importance of the context in which the changed residues operate. Despite the poor complex formation by the human apoE3-Thr-61 mutant, rabbit apoE, which has cysteine at position 112 and threonine at position 61, nevertheless readily forms an SDS-stable complex with Aβ [42]. Even more complicated are the observations with rat apoE, whose capacity to form complexes with Aβ is influenced by the lipoprotein context. Rat apoE does not form complexes when present on VLDL or HDL isolated from rat plasma [42], but a significant amount of complex formation occurs when rat apoE is present on astrocyte HDL-like lipoproteins (M. J. LaDu, C. A. Reardon and G. S. Getz, unpublished work). Rat apoE is similar to apoE4-Thr-61, in that it contains arginine at position 112 and threonine at position 61. However, the human apoE4-Thr-61 mutant is a poor complex former. In addition, it has been shown that delipidation of apoE particles secreted from cultured cells, including the HEK cells used in the present study, decreases the affinity of the apoE isoforms for Aβ and abolishes the isoform differences [43,44]. It appears, then, that not only must apoE be associated with lipid to be capable of complexing with Aβ in an isoform-specific manner, but the particular lipid context may also be influential. Phospholipid differences may also play a role (e.g. phosphatidylethanolamine levels in astrocyte lipoproteins containing the different apoE isoforms [45]). Lipids also affect the ability of apoE to bind to the LDL receptor, which is based critically on a charge–charge interaction between these two molecules. It has recently been shown that the microenvironment of lysine residues 143 and 146 in the receptor binding domain of apoE is significantly modified by its association with lipid [46], and this may explain why apoE needs to be associated with lipid to interact with the low-density lipoprotein receptor. Since apoE4 exhibits a 2-fold preference for VLDL and apoE3 differs in its capacity to complex with Aβ by as much as 20-fold, this suggests that the capacity of the apoE isoforms to form a complex with Aβ may be even more sensitive to the conformational differences between

the isoforms, and that the lipid microenvironment may modify this capacity.

In conclusion, the present results suggest that the nature of the cysteine residue in apoE3 and interactions between the N-terminal and C-terminal domains of human apoE are important for the ability of apoE3 to form an SDS-stable complex with Aβ40. In addition, salt bridges between the N-terminal and C-terminal domains in apoE4 are not directly responsible for its impaired ability to form a complex with Aβ.

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