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Isoform-specific interactions of apolipoprotein E with microtubule-associated protein tau: Implications for Alzheimer disease

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ABSTRACT **The apolipoprotein E (apoE) type 4 allele (*APOE4*) is a susceptibility gene for late-onset familial and sporadic Alzheimer disease. ApoE is found in some neurofibrillary tangle-bearing neurons, one of the major pathologic hallmarks of the disease. Neurofibrillary tangles contain paired helical filaments formed from hyperphosphorylated microtubule-associated protein tau. *In vitro*, tau binds avidly to apoE3, but not to apoE4, forming a bimolecular complex. Tau phosphorylated with a brain extract does not bind either isoform. ApoE3 binds to the microtubule-binding repeat region of tau, which is also the region that is thought to cause self-assembly into the paired helical filament. Binding studies with fragments of ApoE demonstrate that the tau-binding region of apoE3 corresponds to its receptor-binding domain and is distinct from the region that binds lipoprotein particles or β/A4 peptide. Isoform-specific interactions of apoE with tau may regulate intraneuronal tau metabolism in Alzheimer disease and alter the rate of formation of paired helical filaments and neurofibrillary tangles.**

The inheritance of apolipoprotein E4 (*APOE*, gene; apoE, protein) is associated with late-onset familial and sporadic Alzheimer disease (1–3). The allele frequency of *APOE4* is greatly increased in patients in late-onset Alzheimer disease families and is also markedly elevated (0.40 ± 0.026 in patients; 0.16 ± 0.027 in aged-matched controls; $P < 0.00001$) in a series of cases of sporadic Alzheimer disease (2). The increased allele frequency of *APOE4* in Alzheimer disease has been confirmed in many studies from several countries. Corder *et al.* (4) reported that the risk of Alzheimer disease was increased as a function of the inherited dose of *APOE4* and that the mean age of onset ($\bar{x}$) was lowered with each *APOE4* allele ($\bar{x}$ = 68.4 ± 1.2 yr, *APOE4/4*; $\bar{x}$ = 75.5 ± 1.0 yr, *APOE3/4*; $\bar{x}$ = 84.3 ± 1.3 yr, *APOE3/3*) in late-onset families. Inheritance of *APOE2* alleles decreased the risk of disease and increased the age of onset (5), so that *APOE* alleles appear to affect the rate of disease expression (4, 5). ApoE3 is the most common isoform in the general population and contains a single cysteine residue at position 112; apoE4 contains an arginine at this position; additionally apoE2 contains an Arg-158 → Cys difference (6, 7). Isoform-specific properties of apoE have been described and include different binding properties with the low-density lipoprotein receptor, with β/A4 peptide, and with lipoprotein particles (8–10). Also, the single cysteine in apoE3 permits disulfide bond formation with itself or other molecules (refs. 11 and 12; for review, see ref. 7).

In brain tissue from Alzheimer disease patients, apoE is localized to neuritic plaques, vascular amyloid, and some neurofibrillary tangle-bearing neurons (1, 13, 14). *In vitro*, β/A4 peptide binds more avidly to apoE4 than to apoE3 (8). A comparison of brain sections from Alzheimer disease patients homozygous for *APOE4* and *APOE3* has demonstrated an increase in the number of amyloid plaques, the density of β/A4 peptide immunoreactivity, and the area of brain sections occupied by plaques in *APOE4/4* patients (15). Therefore, the well-recognized neuropathological heterogeneity of β/A4 peptide immunoreactivity can be explained by the isoform of apoE inherited by the affected patients. These data suggest that apoE4 decorates β/A4 peptide-containing plaques better than apoE3 *in vivo*.

Dementia in Alzheimer disease is generally accepted to better correlate with the neurofibrillary pathology than with the extent of β/A4 peptide deposition (16). Neurofibrillary lesions contain paired helical filaments, in which the principal constituent is hyperphosphorylated tau, a microtubule-associated protein (17–21). Because of the genetic relevance of *APOE4* and the presence of immunoreactive apoE in neurons containing neurofibrillary tangles, we examined isoform-specific interactions of apoE with recombinant tau before and after phosphorylation with a brain extract.

MATERIALS AND METHODS

Recombinant tau-40 (441-aa tau isoform from human brain) (22) was expressed and purified as described (23). Phosphorylated tau was prepared by incubating recombinant tau-40 with a brain extract, as described (24). Recombinant three-repeat microtubule-binding domain of tau (99-aa protein) was expressed and purified as described (25). Recombinant fragments of apoE3 were prepared and purified as described (26). apoE was purified from individuals homozygous for apoE3 or apoE4, as described (27). The 22-kDa amino-terminal fragment of apoE3 or apoE4 was prepared by thrombin cleavage, followed by purification (28).

Tau-40 (100 μg of protein per ml) and apoE (100 μg of protein per ml) were incubated for 1 hr at 37°C in 20 μl of phosphate-buffered saline, pH 7.30. The incubation was ended by addition of 20 μl of sample buffer without 2-mercaptoethanol, followed by 5 min of boiling. Proteins were electrophoretically separated on a 7.5% polyacrylamide gel and transferred to Immobilon P. The membrane was washed and incubated in primary antibody overnight, as described (8). The primary antibody was monoclonal anti-tau antibody Tau-1 (Boehringer Mannheim) at a dilution of 1:2000 in

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Abbreviations: β/A4, amyloid β; apoE, apolipoprotein E.
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Blotto. After washing, the membrane was incubated for 1 hr with goat anti-mouse $F(ab')_2$ conjugated with horseradish peroxidase (1:1500) (Boehringer Mannheim). After being washed the horseradish peroxidase was visualized with an enhanced chemiluminescence detection kit (Amersham) and exposed to Hyperfilm ECL (Amersham) (8). The primary antibody used to detect the recombinant three-repeat microtubule-binding domain of tau was rabbit antiserum 135 (22) at a dilution of 1:5000.

RESULTS

In vitro purified human apoE3 bound to tau-40, forming a molecular complex that resisted dissociation by boiling in 2% SDS. Fig. 1 (lane 2, lower arrow) shows that the apoE3/tau complex had an apparent molecular mass of ≈105 kDa (tau-40, 68 kDa; apoE, 34 kDa) and was not observed in either protein preparation alone. Tau also bound the disulfide-linked apoE3 homodimer (Fig. 1, lane 2, upper arrow). Binding of tau and apoE3 was maximal within 30 min at 37°C and occurred between pH 4.6 and 7.6. Binding of tau to apoE3 could be detected down to 3×10^{-8} M apoE. The apoE3/tau complex was dissociated by boiling in the reducing agent 2-mercaptoethanol (Fig. 1). However, the apoE3/tau was probably *not* complexed through disulfide bond formation because tau bound both the monomer and the homodimer of apoE3 (Fig. 1). There was only insignificant binding of tau by apoE4 under a variety of conditions, including increased duration of incubation, increased concentration of apoE4, or pH values 7.6–4.6 (Fig. 2).

ApoE (299 residues) contains two functionally important domains, one that binds the low density lipoprotein receptor and the other that binds lipoprotein particles (very low density lipoprotein or high density lipoprotein). Thrombin cleaves apoE at residues 191 and 215, yielding a 22-kDa amino-terminal fragment and a 10-kDa carboxyl-terminal fragment (28, 29). The receptor-binding domain is located within the 22-kDa amino-terminal fragment (28, 29), and both the lipid-binding (30) and the β/A4 peptide-binding regions (8) are within the 10-kDa fragment. Tau bound to the 22-kDa amino-terminal fragment of apoE3 (Fig. 1). Recombinant apoE3 fragments 1–244, 1–266, and 1–272 bound equivalent amounts of tau when compared with that bound by the 22-kDa fragment of native apoE (aa 1–191) (Fig. 3). Thus, tau binds to the fragment of apoE3 which also binds the low

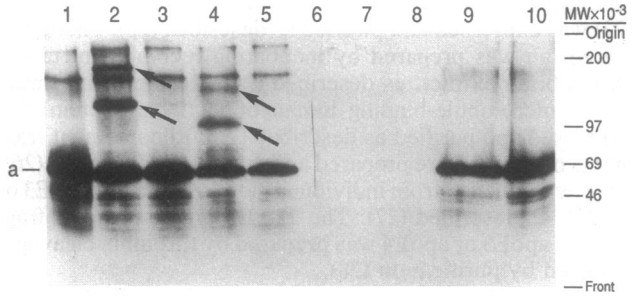

FIG. 1. Demonstration of binding of recombinant tau by purified native apoE3, detected by an anti-tau antibody. Lanes: 1, Tau-40 alone; 2, Tau-40 and apoE3; 3, Tau-40 and apoE4; 4, Tau-40 and 22-kDa amino-terminal fragment of native apoE3; 5, Tau-40 and 22-kDa fragment of native apoE4; 6, apoE3 alone; 7, apoE4 alone; 8, Blank; 9, Tau-40 and apoE3, incubated and then boiled in sample buffer containing 0.2% 2-mercaptoethanol; 10, Tau-40 and apoE4, incubated and then boiled in sample buffer containing 0.2% 2-mercaptoethanol. Lane 2 upper arrow indicates the apoE3 homodimer/tau complex, and the lower arrow indicates the apoE3 monomer/tau complex. Lane 4 upper arrow indicates the 22-kDa apoE3 fragment homodimer/tau complex, and the lower arrow indicates the 22-kDa apoE3 fragment monomer/tau complex. a, Unbound tau.

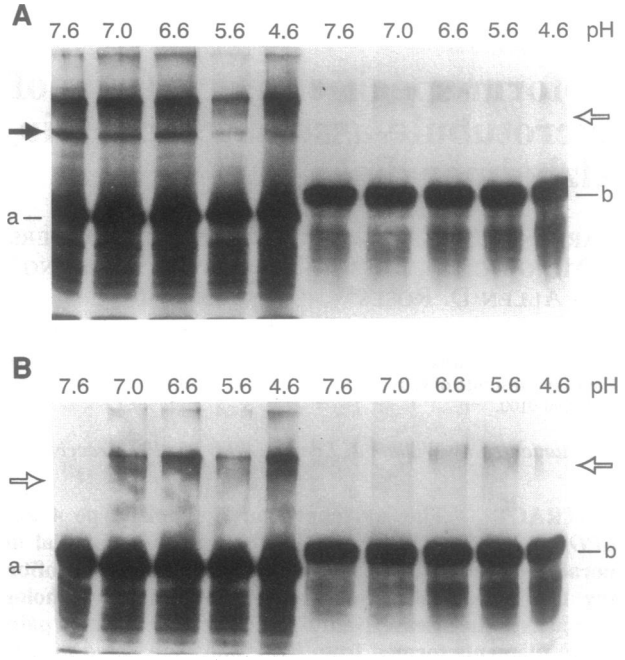

FIG. 2. pH-independent binding of recombinant tau to native apoE3 and absence of binding to apoE4; absence of binding of hyperphosphorylated tau to either apoE3 or apoE4. ApoE3 (*A*) or apoE4 (*B*) was incubated with either tau-40 (lanes 1–5) or hyperphosphorylated tau-40 (lanes 6–10) in citric acid/Na_2HPO_4 buffer (13) at the indicated pH for 30 min at 37°C. Closed arrow, apoE3/tau complex; open arrows, predicted positions of apoE4/tau or apoE/P-tau complexes. Note the slower migration of hyperphosphorylated tau (labeled b), compared with nonphosphorylated tau (labeled a).

density lipoprotein receptor (28, 29). This region is distinct from the region (between aa 245–272) that binds lipoprotein particles (25) and the β/A4 peptide (8). The 22-kDa amino-terminal fragment of apoE4 does not bind tau. (Fig. 1).

Paired helical filament tau is phosphorylated at a number of serine/threonine-proline sites (31, 32). At least some of these sites are phosphorylated by incubating recombinant tau with a crude rat brain extract and ATP (24). Recombinant tau-40 phosphorylated in this manner did not bind either apoE3 or apoE4 (Fig. 2), even with a prolonged 12-hr incubation. Failure of apoE3 and apoE4 to bind to tau phosphorylated by brain extract resulted from phosphorylation because omission of ATP from the incubation mixture still permitted binding of apoE3 to the nonphosphorylated tau

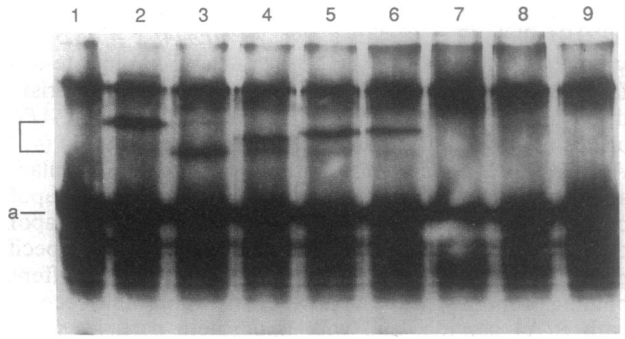

FIG. 3. Binding of apoE3 fragments to tau. Lanes: 1, Tau-40 alone; 2, Tau-40 and native apoE3 (aa 1–299); 3, Tau-40 and 22-kDa amino-terminal fragment of native apoE3 (aa 1–191); 4, Tau-40 and recombinant apoE3 (aa 1–244); 5, Tau-40 and recombinant apoE3 (aa 1–266); 6, Tau-40 and recombinant apoE3 (aa 1–272); 7, Tau-40 alone; 8, Tau-40 and native apoE4 (aa 1–299); 9, Tau-40 alone. a, Tau-40 at 68 kDa; bracket, tau bound to the apoE3 fragments.

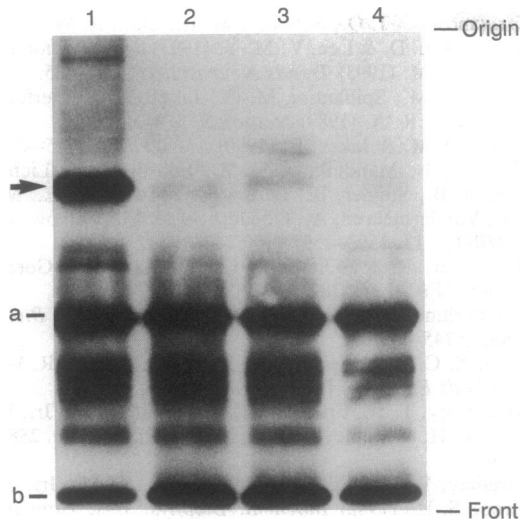

FIG. 4. Binding of native apoE3 to the microtubule-binding repeat region of tau. Lanes: 1, apoE3 and microtubule-binding repeats of tau; 2, apoE3 and microtubule-binding repeats of tau; apoE3 was boiled 5 min before incubation; 3, apoE4 and microtubule-binding repeats of tau; 4, apoE4 and microtubule-binding repeats of tau; apoE4 was boiled 5 min before incubation. arrow, Complex of apoE3 with microtubule-binding repeat of tau; a, homodimer of microtubule-binding repeat of tau (27 kDa); b, monomer of microtubule-binding repeat of tau (12 kDa).

(data not shown). In Alzheimer disease hyperphosphorylated tau is believed to self-assemble into the paired helical filament by formation of antiparallel dimers of the microtubule-binding repeat region (33). *In vitro*, the microtubule-binding repeats of tau self-assemble into paired helical-like filaments (25, 33). As shown in Fig. 4, apoE3 bound to the microtubule-binding repeat region of tau, whereas apoE4 did not bind. Boiling apoE3 before incubation with the microtubule-binding repeats prevented binding, demonstrating conformational requirements for binding.

DISCUSSION

The present experiments demonstrate that *in vitro* apoE3 binds to recombinant tau protein, whereas apoE4 shows no significant binding. Interestingly, neither isoform binds to tau protein phosphorylated with a rat brain extract. The interaction between apoE3 and tau is mediated through the low density lipoprotein receptor-binding domain of apoE3 and the microtubule-binding domain of tau, respectively. These *in vitro* interactions form the basis of a testable hypothesis postulating that apoE3 plays a protective role in the formation of paired helical filaments in Alzheimer disease. Binding of apoE3 or apoE2 to tau may interfere with the hyperphosphorylation of tau and/or its self-assembly into paired helical filaments. In a disease that takes many years to become symptomatic, small apoE isoform-specific effects on the rate of formation, or metabolism, of hyperphosphorylated tau may be significant. Individuals who inherit a single or double dose of *APOE4* could produce more hyperphosphorylated tau and/or form paired helical filaments faster than individuals who inherit *APOE3* alleles. The neurofibrillary tangle may be another phenotypic manifestation of a process that occurs when tau metabolism is altered. The number of neurons containing neurofibrillary tangles dying at a particular time in Alzheimer disease therefore need not correlate with the *APOE* genotype (34).

A necessary condition for a protective action of apoE3 in Alzheimer disease is that tau and apoE3 colocalize in the nerve cells that are prone to neurofibrillary tangle formation. *In situ* hybridization studies on brain have indicated that apoE mRNA is produced by astrocytes (35). However, recent immunohistochemical studies on Alzheimer disease brain have shown the presence of apoE in both tangle-bearing and tangle-free nerve cells, indicating that apoE may play a role in neuronal degeneration or regeneration in the central nervous system (1, 34, 36). In a series of 24 patients with Alzheimer disease, immunoreactive apoE was demonstrated in hippocampal neurons independent of *APOE* genotype (34). ApoE was also observed in hippocampal granule cells of seven out of seven Parkinson disease patients, several patients with other neurodegenerative diseases, and two out of six aged controls without known brain disease. Only a small proportion of the apoE immunoreactive neurons in Alzheimer disease patients also contained neurofibrillary tangles (34). ApoE immunoreactive cortical neurons were commonly observed in aged rats and prosimians (*Otolemur*) but were not seen in mice or baboons. ApoE immunoreactivity defined specific subsets of neurons, with punctate staining around soma, diffuse cytoplasmic staining, and granular staining. Ultrastructural studies of human and prosimian brain confirmed the cytoplasmic localization of apoE immunoreactivity (34), as have recent studies on human inclusion body myositis (37).

The exact mechanisms underlying the entry of apoE into the cytoplasmic compartment of nerve cells are not understood but may involve endosomal pathways. An analogous situation has recently been reported in skeletal muscle from patients with sporadic and inherited forms of inclusion body myositis (37). This disease demonstrates remarkable similarities to the neuropathology of Alzheimer disease. Thus, in muscle tissue from affected individuals, one finds intracellular amyloid fibrils made of B/A4 peptide and paired helical-like filaments (tubulofilaments) made of hyperphosphorylated tau (38, 39). Moreover, the muscle cells also contain cytoplasmic apoE, even though no evidence suggests that muscle cells produce apoE (37). Interestingly, apoE is found in close proximity to the paired helical-like filaments (37). These various lines of evidence indicate that in both Alzheimer disease and inclusion body myositis apoE gains access to the cytoplasmic compartment of nerve cells and muscle cells. The sorting mechanisms responsible for the intracellular trafficking of apoE may also be responsible for the sorting of the amyloid precursor protein with specific mutations associated with some cases of early-onset Alzheimer disease (40).

ApoE participates in lipoprotein particle uptake mediated by cell surface receptors, binds B/A4 peptide (1, 14, 41), and serves in regeneration and degeneration in the central and peripheral nervous systems. We show here that apoE3, but not apoE4, interacts with tau protein *in vitro*. These differences in the interactions of apoE3 and apoE4 isoforms are attributable to a single-amino acid substitution (Cys-112 → Arg). Inheritance of *APOE4* is genetically associated with increased risk and younger onset of Alzheimer disease. Further *in vitro* studies in tissue culture systems and *in vivo* studies in transgenic animal models will be necessary to characterize isoform-specific, time-dependent reactions favoring the formation of paired helical filaments. Taking into account prior observations of widespread intracellular localization of apoE in endosomes, peroxisomes, and cytoplasm in rat hepatocytes (42) and current observations in human neurons (1, 34) and muscle (37), we suggest an isoform-specific role for apoE in intracellular metabolism related to paired helical filament formation. The differences in tau binding to apoE3 and apoE4 present opportunities for testing the role of inherited risk factors in the rate of development of Alzheimer disease. Understanding the intraneuronal metabolism of apoE may lead to the design and testing of rational neuroprotective treatments for late-onset Alzheimer disease.

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