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Transthyretin sequesters amyloid β protein and prevents amyloid formation

(sequestration)

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ABSTRACT **The cardinal pathological features of Alzheimer disease are depositions of aggregated amyloid β protein (Aβ) in the brain and cerebrovasculature. However, the Aβ is found in a soluble form in cerebrospinal fluid in healthy individuals and patients with Alzheimer disease. We postulate that sequestration of Aβ precludes amyloid formation. Failure to sequester Aβ in Alzheimer disease may result in amyloidosis. When we added Aβ to cerebrospinal fluid of patients and controls it was rapidly sequestered into stable complexes with transthyretin. Complexes with apolipoprotein E, which has been shown to bind Aβ *in vitro*, were not observed in cerebrospinal fluid. Additional *in vitro* studies showed that both purified transthyretin and apolipoprotein E prevent amyloid formation.**

Amyloid β protein (Aβ) is a normal 4-kDa derivative of a large transmembrane glycoprotein, amyloid β precursor protein (APP). Aβ is found in an aggregated, poorly soluble form in extracellular amyloid depositions in the brains and leptomeninges of patients with Alzheimer disease (AD), Down syndrome, and hereditary cerebral hemorrhage with amyloidosis, Dutch type (1–3). In contrast, it is found in a soluble form in human cerebrospinal fluid (CSF), in serum, and in the conditioned media of many different cultured cell types (4, 5). A number of studies with synthetic Aβ *in vitro* have shown that this peptide aggregates easily and forms amyloid fibrils similar to the fibrils found in the brains of patients with AD and Down syndrome (3, 6–8). The mechanism by which this normally produced peptide forms amyloid is unknown. It is also not clear why highly aggregating Aβ does not form amyloid in extracellular fluids of patients with AD and healthy individuals.

We recently postulated that Aβ is sequestered by extracellular proteins, thereby preventing amyloidosis (9). Failure of this mechanism in AD patients could lead to amyloid formation. A sequestration mechanism implies, first, the existence of sequestering proteins that interact with and bind Aβ and, second, sequestered Aβ cannot aggregate to form amyloid.

Several extracellular proteins have been identified that bind immobilized Aβ. These include apolipoprotein E (apoE), apolipoprotein J, and APP, all of which are found in CSF (10–12). The binding of apoE, the major CSF apolipoprotein, is particularly relevant to late-onset familial and sporadic AD. Patients homozygous for *APOE4* have more amyloid depositions than patients homozygous for *APOE3* (13). The inheritance of *APOE4* allele also increases the risk and decreases the age of onset of the disease significantly (14). Thus, apoE appeared to be a candidate for sequestration of Aβ. However, we now report the identification of a CSF protein that rapidly forms complexes with Aβ, prevents formation of amyloid fibrils, and is distinct from apoE.

MATERIALS AND METHODS

Analysis of CSF Proteins Forming Complexes with ^{125}I-Labeled Aβ (^{125}I-Aβ). Synthetic $A\beta_{1-28}$ and $A\beta_{1-40}$ were obtained from Bachem. Samples of CSF were obtained from the Department of Neurology, State University of New York, Stony Brook, and the Division of Neurology, Duke University Medical Center. Iodination of $A\beta_{1-28}$ and $A\beta_{1-40}$ was performed using iodinated (^{125}I) Bolton–Hunter reagent from Amersham according to the manufacturer's instructions. Ten microliters of human CSF was incubated with 10^5 dpm of ^{125}I-$A\beta_{1-28}$ (specific activity, 3–6 × 10^6 dpm/μg) in a final volume of 20 μl of phosphate-buffered saline (PBS) at pH 7.4 at 37°C. Samples were analyzed by SDS/PAGE. A 12% Tris-glycine gel was used for analysis of complexes Aβ with CSF proteins and a 13% gel in Tricine buffer was used for analysis of Aβ aggregation. The gel was then dried and exposed to an x-ray X-Omat film from Kodak. The purification of Aβ binding activity was monitored using ^{125}I-$A\beta_{1-28}$ and electrophoresis in SDS/PAGE gel and included three steps. Step 1: 5 ml of CSF was subjected to ion-exchange chromatography on a 5-ml column with DEAE-Sepharose equilibrated with 50 mM Tris·HCl (pH 7.4). The peak of Aβ binding activity was eluted at 0.4 M NaCl. Step 2: the peak fractions were combined, diluted five times, and further passed through a heparin-Sepharose column in 50 mM NaCl/50 mM Tris·HCl, pH 7.4. The Aβ binding activity appeared in unbound fractions. Step 3: the combined fractions containing Aβ binding activity were chromatographed on a FPLC-mono Q column with a gradient of 0.1–0.3 M NaCl in 50 mM Tris·HCl (pH 7.4). Two hundred micrograms of purified protein was concentrated on a Speed Vac concentrator and then reduced and alkylated. The purified protein was digested with trypsin, and the peptides were separated by reverse-phase HPLC. The two largest peptides were sequenced by automated Edman degradation with an Applied

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Abbreviations: AD, Alzheimer disease; Aβ, amyloid β protein; APP, amyloid β precursor protein; CSF, cerebrospinal fluid; TTR, transthyretin; apoE, apolipoprotein E; BSA, bovine serum albumin.

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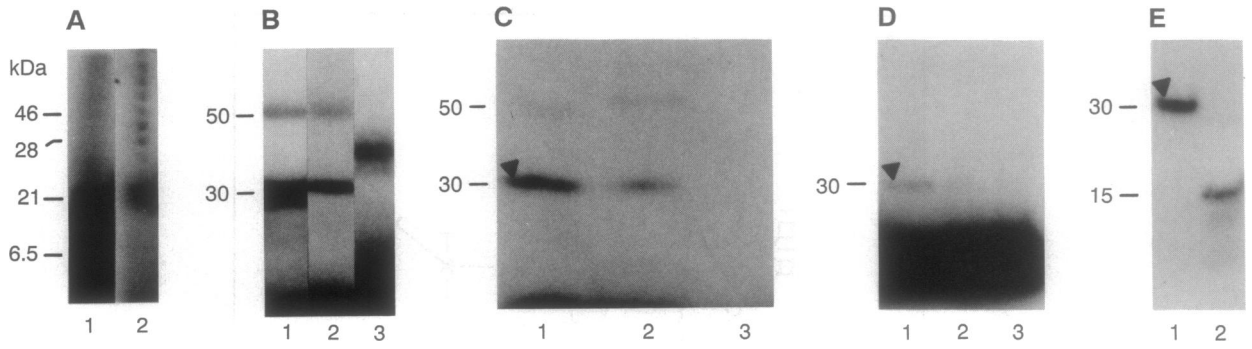

FIG. 1. Analysis of ^{125}I-Aβ complexes with CSF proteins. (*A*) Aggregates of ^{125}I-Aβ_{1-28} formed after incubation for 24 hr (lane 1) and pelleted by centrifugation at 15,000 × *g* for 10 min through a 20% sucrose cushion (lane 2). (*B*) Complexes of ^{125}I-Aβ_{1-28} with CSF proteins formed after incubation for 24 hr (lane 1) and 10 min (lane 2); complexes of ^{125}I-Aβ_{1-28} with human apoE3 formed after incubation for 24 hr (lane 3). (*C*) Competition of complex formation of ^{125}I-Aβ_{1-28} with CSF proteins by unlabeled Aβ_{1-28}: CSF (lane 1), CSF plus a 10-fold excess of unlabeled Aβ_{1-28} (lane 2), and CSF plus a 200-fold excess of unlabeled Aβ_{1-28}, ▼, A 30-kDa band. (*D*) Competition of complex formation of ^{125}I-Aβ_{1-28} with TTR (Calbiochem) by unlabeled Aβ_{1-40}. Radiolabeled Aβ was incubated with 0.1 μM TTR (lane 1), 0.1 μM TTR plus a 100-fold excess of unlabeled Aβ_{1-40} (lane 2), and 0.1 μM TTR plus a 500-fold excess of unlabeled Aβ_{1-40} (lane 3). ▼, A 30-kDa band. (*E*) Analysis of complexes of ^{125}I-Aβ_{1-28} with TTR under different conditions. Before electrophoresis the samples were incubated in Laemmli buffer without 2-mercaptoethanol for 5 min at room temperature (lane 1) or were boiled for 10 min in a Laemmli buffer with 0.2 M 2-mercaptoethanol (lane 2). ▼, A 30-kDa band. Similar results were obtained for binding of TTR with radiolabeled Aβ_{1-40} (data not shown).

Biosystems 477A sequencer with on-line phenylthiohydantoin analysis using Applied Biosystems 120A HPLC. For Western blot analysis, 10 μg of Aβ_{1-40} peptide was incubated in 40-μl samples overnight at 37°C in PBS (pH 7.2) and separated by SDS/PAGE; the proteins were transferred onto membranes. Rabbit anti-Aβ antibody SGY2134 was kindly provided by Steven G. Younkin (Case Western Reserve University). Sheep anti-transthyretin (TTR) antibody was purchased from ICN. Human apoE3 and apoE4 were isolated from human plasma as described (15). Bovine serum albumin (BSA) was obtained from Sigma. TTR was obtained from Calbiochem. Immunoreactive proteins were detected by the ECL method (Amersham).

Thioflavin-T-Based Fluorometric Assay. Synthetic Aβ_{1-28} at 300 μM was mixed with the indicated concentrations of proteins, and aggregation was initiated with 100 mM sodium acetate (pH 5.2). After 18 hr, 5-μl samples were mixed with 10 μM thioflavin-T in 50 mM potassium phosphate (16), and arbitrary fluorescence units were measured at 450-nm excitation and 482-nm emission on a Perkin–Elmer LS-50 fluorimeter.

Congo Red Staining. Synthetic Aβ_{1-28} at 300 μM was mixed with the indicated concentrations of proteins, and aggregation was initiated with 100 mM sodium acetate (pH 5.2). After 18 hr, samples were mixed with 0.2% Congo red in 100 mM sodium acetate (pH 5.2), and 5 μl was spotted onto a microscope slide. Slides were viewed under polarized light at a magnification of 200.

Electron Microscopy. Aβ_{1-28} (100 μg/ml) was dissolved in water, sonicated for 15 sec, added to proteins, incubated for 16 hr at 37°C in PBS (pH 7.2), and stained with 2% uranyl acetate. Samples were examined and photographed at a magnification of 25,000 on a Hitachi-12 electron microscope.

Molecular Modeling. The coordinates for TTR were kindly provided by Jean A. Hamilton and Larry K. Steinrauf (Department of Biochemistry, Indiana University School of Medicine). The TTR structure had been determined originally by x-ray crystallography (17). The coordinates for Aβ_{1-28} correspond to the solution structure as determined by two-dimensional NMR and distance geometry/simulated annealing (18). The electrostatic potential was calculated and displayed at and around TTR and Aβ_{1-28} with the program GRASP (Anthony Nichols, Kim Sharp, and Barry Honig, Columbia University). These calculations were based on the linearized form of the Poisson–Boltzmann equation assuming that the dielectric constant of the solution is 80 and that of the intramolecular space is 2. These calculations were done for a 0.1 M NaCl solution. The values for the partial charges for the amino acids were those of the AMBER force field (Peter Kollman, University of California, San Francisco).

RESULTS

To identify the proteins interacting with Aβ in human CSF, we used synthetic Aβ_{1-28} and Aβ_{1-40} labeled by an iodinated Bolton–Hunter reagent. Radioiodinated Aβ forms aggregates in physiological buffers (Fig. 1*A*). When radiolabeled Aβ_{1-28} or Aβ_{1-40} was added to CSF samples, instead of aggregates, two bands with apparent molecular masses of 30 and 50 kDa were observed (Fig. 1*B*). These bands were distinct from the 40-kDa apoE–Aβ complexes (Fig. 1*B*, lane 3) that were previously described (10). The formation of radiolabeled Aβ complexes in CSF could be specifically competitively inhib-

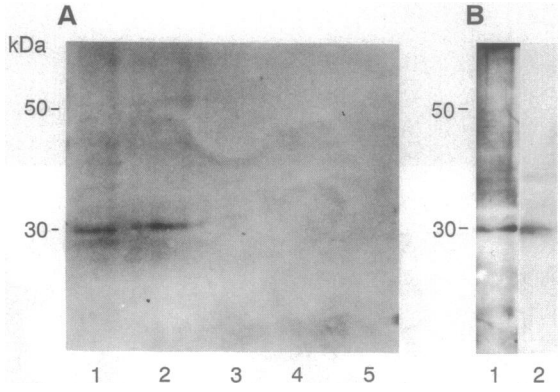

FIG. 2. Western blot analysis of TTR–Aβ complexes. (*A*) Ten micrograms of Aβ_{1-40} peptide was incubated in 40-μl samples overnight at 37°C in PBS (pH 7.2) with 10 μl of CSF (lane 1), 1 μg of TTR (lane 2), or 50 μg of BSA (lane 3). Ten microliters of CSF (lane 4) and 1 μg of TTR (lane 5) in 40-μl samples without Aβ were used as controls for cross-reactivity in immunostaining. The samples were analyzed by SDS/PAGE and immunoblotting using rabbit anti-Aβ antibody SGY2134 (3). (*B*) Ten micrograms of Aβ_{1-28} peptide plus 10 μl of CSF were incubated in a 40-μl sample overnight at 37°C in PBS (pH 7.2). The sample was analyzed by SDS/PAGE and immunoblotting. After the transfer, the membrane was cut lengthwise in two strips and immunostained with two different antibodies. Lane 1, immunostaining with anti-Aβ antibody SGY2134; lane 2, immunostaining with anti-TTR antibody.

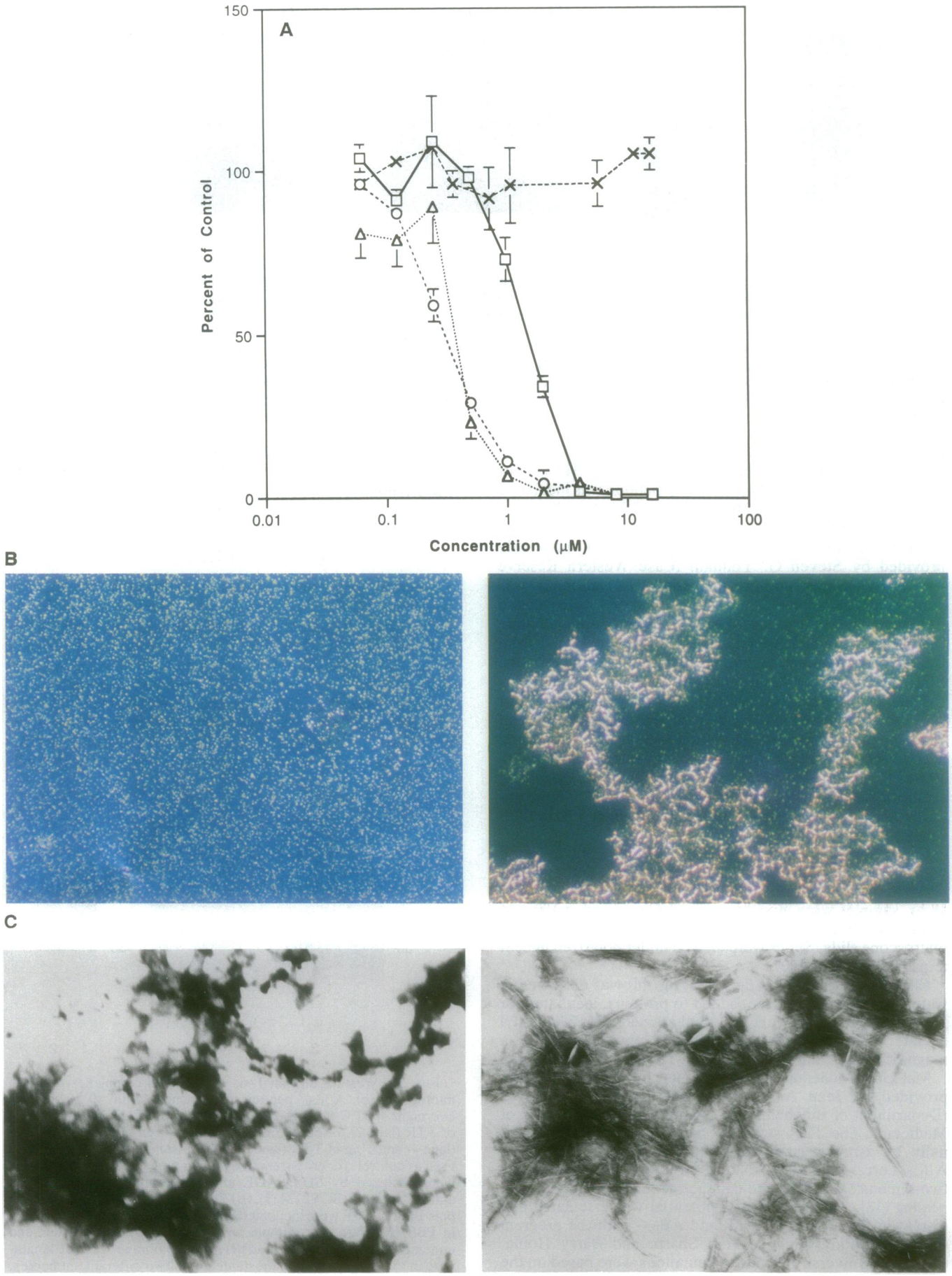

FIG. 3. *(Legend appears at the bottom of the opposite page.)*

ited with unlabeled Aβ (Fig. 1C). The CSF protein that formed a 30-kDa complex with Aβ was purified and subjected to trypsin digestion, and the two largest peptides were sequenced. These sequences ALGISPFHEHAEVVFTANDSGP and RYTIAALLISPYSYSTTAVVINPK perfectly matched amino acid residues 81–102 and 104–127, respectively, of TTR, a transporter of thyroxine and vitamin A in the brain (19, 20). Commercial human plasma TTR also formed 30-kDa complexes with $A\beta_{1-28}$ that could be competitively inhibited with unlabeled $A\beta_{1-40}$, confirming the specificity of binding (Fig. 1D).

TTR is a homotetrameric protein with 127 amino acid residues in each chain. TTR dissociates to form 30-kDa dimers in SDS and 15-kDa monomers after boiling in SDS with reducing agents (21). The 30-kDa TTR–Aβ complexes appeared as 15-kDa complexes after boiling in SDS with reducing agents, suggesting that TTR monomer binds Aβ (Fig. 1E).

Using similar analytical techniques, we identified the CSF protein that formed the 50-kDa complex with radiolabeled Aβ as albumin.

We then determined that TTR is the major Aβ binding protein in CSF. Unlabeled Aβ was incubated with CSF, TTR, or albumin, and the complexes were analyzed using Western blot techniques with anti-TTR and anti-Aβ antibodies. Under nonreducing conditions, only TTR–Aβ complexes with an apparent molecular mass of 30 kDa were observed (Fig. 2). Complexes of Aβ with commercial bovine serum albumin or with albumin in human CSF were not detected. Thus, radioiodination of Aβ by Bolton–Hunter reagent may cause the nonspecific binding of radiolabeled peptide to albumin.

The effect of Aβ binding proteins on aggregation of unlabeled $A\beta_{1-28}$ was tested by a quantitative thioflavin-T fluorometric assay (16). It has been suggested that apoE may promote amyloid formation (22). Therefore, in addition to TTR, two isoforms of apoE and albumin were tested. TTR, apoE3, and apoE4 reduced the fluorescence signal, indicating the prevention of $A\beta_{1-28}$ aggregation, while albumin had no effect (Fig. 3A). Inhibition of $A\beta_{1-28}$ aggregation was dose dependent with a 50% reduction in signal observed at 1.4 μM for TTR and 0.4 μM for apoE3 or apoE4.

When amyloid found in patient tissues or aggregated synthetic Aβ is stained with Congo red, it produces a specific green-to-yellow birefringence when viewed under polarized light (1, 3). We found that the addition of albumin prior to aggregation of $A\beta_{1-28}$ did not prevent the appearance of birefringence (Fig. 3B *Right*). In contrast, when TTR or apoE was added to $A\beta_{1-28}$ prior to aggregation, fewer or no characteristic aggregates producing birefringence were observed (Fig. 3B *Left*).

Another feature of amyloid is the formation of fibrils with a characteristic electron microscopic pattern (1, 3). Synthetic $A\beta_{1-28}$ readily forms these typical 5- to 10-nm-thick amyloid fibrils (Fig. 3C *Right*). When Aβ was incubated with TTR, only amorphous masses with few abortive short fibrils were observed, suggesting that TTR prevented formation of characteristic fibrils (Fig. 3C *Left*). apoE3 or apoE4 had the same effect as TTR (data not shown).

To understand the interaction of Aβ and TTR, we built a model of the TTR–Aβ complex. The molecular surfaces of Aβ and TTR were generated, and electrostatic potentials were calculated with the program GRASP on a Silicon Graphics (Mountain View, CA) Iris. Coordinates for $A\beta_{1-28}$ correspond to the solution structure as determined by two-dimensional NMR and distance geometry/simulated annealing (18), while coordinates for TTR structure had been determined by x-ray crystallography (17). It is strikingly clear that the electrostatic potentials of the α-helical $A\beta_{1-28}$ are very dipolar in nature (Fig. 4A). Likewise, TTR has clearly demarcated regions that spawn negative (purple) or positive (yellow) electrostatic potentials (Fig. 4B).

To find the best fit between TTR and Aβ, we tried to (*i*) maximize surface contacts, (*ii*) maintain the relative orientations to enhance electrostatic attraction, (*iii*) bind the amyloid peptide to each subunit of TTR independently, and (*iv*) avoid the TTR monomer surface involved in tetramer formation. Within these constraints we suggest the best binding scheme, which is shown in Fig. 4C, with TTR subunits colored blue and green and two $A\beta_{1-28}$ molecules colored yellow. The concave, positive potential inducing surface of $A\beta_{1-28}$ containing the residues Arg (5), His (13), Lys (16), and Lys (28) matches remarkably well with the convex, negative potential inducing surface on TTR containing the residues Asp (38, 165), Glu (42, 169), Glu (62, 189), and Glu (66, 193).

DISCUSSION

Our experiments clearly show that sequestered Aβ cannot participate in amyloid fibril formation. Although TTR is not the only protein that binds Aβ (10–12), it is the major Aβ sequestering protein in human CSF. The concentration of TTR is two orders of magnitude greater than concentration of Aβ and is higher than the concentration of other known Aβ binding proteins in CSF. The approximate concentrations are 3 nM for Aβ, 2 μM for albumin, 0.3 μM for TTR, 0.1 μM for apoE, 0.03 μM for apoJ, and 0.03 μM for APP (4, 23–26). Our data do not exclude the possibility that other proteins form complexes with Aβ. However, most, if not all, Aβ is probably sequestered by TTR (Figs. 1 and 2).

The biological activity of Aβ has been demonstrated in a number of experiments with cell cultures (1, 27–30). Binding of TTR and other proteins to Aβ may regulate its biological activity and play a role in the transport and clearance of the peptide. Therefore, sequestration of Aβ may represent a key step in a homeostatic mechanism that is responsible for both the control of biological activity and the clearance of Aβ.

The identification of Aβ binding proteins suggests that prevention of Aβ aggregation and amyloid formation requires a dynamic equilibrium of multiple extracellular factors participating in the sequestration of Aβ. A change in the synthesis of a number of proteins in AD brain resembles a typical picture of acute-phase response. A decreased level of TTR in CSF (31, 32) and increased levels of Aβ (33), ApoE (34), ApoJ (35), and APP (36) in the brains of AD patients may represent such a change, alter the existing equilibrium of proteins that normally bind Aβ, and facilitate amyloid formation. Thus, amyloid forms when sequestration fails.

Over 40 mutations have been documented in TTR, and some lead to TTR amyloid formation in familial amyloidotic polyneuropathy (19, 20). Variants of TTR could be associated with AD in families not linked to defined genetic loci on chromosomes 14, 19, and 21 (1, 2) or modulate the effect of other genes. The suggested structure of TTR–Aβ complex, furthermore, can provide a molecular basis for the design of drugs that could prevent amyloid formation.

FIG. 3 (*on opposite page*). Prevention of aggregation of Aβ. (*A*) Thioflavin-T-based fluorometric assay of $A\beta_{1-28}$ aggregation in the presence of different concentrations of BSA (X), TTR (□), apoE3 (△), and apoE4 (○). One hundred percent aggregation equals the average fluorescence signal of Aβ. Each point represents the average of quadruplicate measurements; points are plotted as percentages with standard errors for the given concentrations. (*B*) Congo red staining of Aβ aggregates in the presence of 5 μM BSA (*Right*) or 3 μM TTR (*Left*). (*C*) Electron micrographs of $A\beta_{1-28}$ aggregates without (*Right*) or with (*Left*) 2 μM TTR. (Bar = 100 nm.)

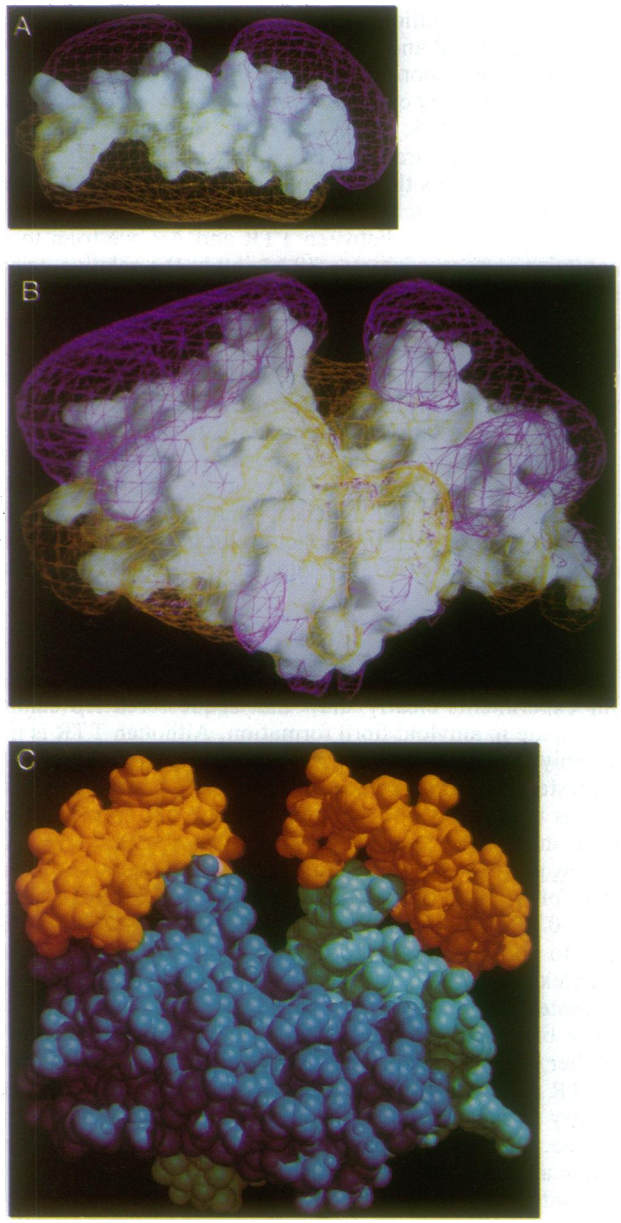

FIG. 4. Computer graphic models of Aβ, TTR, and TTR–Aβ complex. (*A* and *B*) Aβ (*A*) and TTR dimer (*B*) molecular surfaces are shown in white; electrostatic potentials were calculated with the program GRASP on a Silicon Graphics Iris. The -1 *kT* potential contour is shown in purple and the $+1$ *kT* potential contour is shown in yellow. (*C*) TTR–$A\beta_{1-28}$ complex as a space filling model. For clarity, monomers of TTR dimer are shown in blue and green. Two Aβ molecules are shown in yellow. The following amino acid residues were found on the contacting surface of Aβ: Arg (5), Ser (8), Gly (9), Val (12), His (13), Gln (15), Lys (16), Phe (19), Phe (20), Asp (23), Val (24), Asn (27), and Lys (28). The following amino acid residues were found on the contacting surface of TTR dimer: Arg (34, 161), Ala (37, 164), Asp (38, 165), Thr (40, 167), Glu (42, 169), Glu (62, 189), Val (65, 192), and Glu (66, 193). The first number in parentheses is the residue number for the first subunit and the second number is for the second subunit of TTR.

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