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Retinol-Binding Protein Interferes with Transthyretin-Mediated β -Amyloid Aggregation Inhibition

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Abstract

β -Amyloid ($A\beta$) aggregation is causally linked to Alzheimer's disease. On the basis of *in vitro* and transgenic animal studies, transthyretin (TTR) is hypothesized to provide neuroprotection against $A\beta$ toxicity by binding to $A\beta$ and inhibiting its aggregation. TTR is a homotetrameric protein that circulates in blood and cerebrospinal fluid; its normal physiological role is as a carrier for thyroxine and retinol-binding protein (RBP). RBP forms a complex with retinol, and the holoprotein (hRBP) binds with high affinity to TTR. In this study, the role of TTR ligands in TTR-mediated inhibition of $A\beta$ aggregation was investigated. hRBP strongly reduced the ability of TTR to inhibit $A\beta$ aggregation. The effect was not due to competition between $A\beta$ and hRBP for binding to TTR, as $A\beta$ bound equally well to TTR–hRBP complexes and TTR. hRBP is known to stabilize the TTR tetrameric structure. We show that $A\beta$ partially destabilizes TTR and that hRBP counteracts this destabilization. Taken together, our results support a mechanism wherein TTR-mediated inhibition of $A\beta$ aggregation requires not only TTR– $A\beta$ binding but also destabilization of TTR quaternary structure.

Graphical Abstract

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The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.8b00517.

Fluorescence spectra confirming retinol binding, FRET schematics, $A\beta$ fibrillogenesis kinetic analysis, DLS analysis, TEM images of the TTR–hRBP complex with $A\beta$, FRET analysis of binding of $A\beta$ oligomers to TTR tetramers, RBP– $A\beta$ aggregation and binding data, and extended period TTR–hRBP– $A\beta$ gel assay (PDF)

The characteristic features of Alzheimer's disease (AD) include extracellular senile plaques, intraneuronal neurofibrillary tangles, and extensive neuronal cell death. The plaques contain insoluble amyloid deposits composed predominantly of the peptide β -amyloid ($A\beta$), produced by cleavage of the transmembrane amyloid precursor protein (APP).¹ Upon release from APP, $A\beta$ monomers spontaneously self-assemble into soluble oligomers and insoluble fibrillar aggregates,^{2,3} a process that is believed to be causally linked to neurotoxicity.^{4,5}

Transgenic mice expressing the Swedish mutation APP_{Sw} (Tg2576) produce high levels of $A\beta$ and develop amyloid deposits.⁶ However, these mice lack neurofibrillary tangles and, although gliosis and dystrophic neuritis are observed, there is no neuronal cell loss,⁷ seemingly contradicting the hypothesis that $A\beta$ aggregation is responsible for AD pathology. This result was explained by the demonstration of an increased level of transthyretin (TTR) expression in Tg2576 mice compared to controls.⁸ Administration of the anti-TTR antibody led to increased levels of tau phosphorylation and neuronal cell death.⁹ This result gave rise to the hypothesis that an increased level of TTR expression was a protective response against high levels of $A\beta$, a hypothesis that is supported by other animal studies.^{10–15} TTR's ability to abrogate $A\beta$ toxicity in cell culture has been demonstrated in numerous studies.^{16–19} Beyond cellular and animal studies, there is evidence for a role of TTR in human AD; for example, amyloid deposits in AD brain are strongly stained by TTR.¹⁸ TTR levels in cerebrospinal fluid (CSF) fluctuate with disease state,^{20–27} suggesting that TTR levels respond dynamically to $A\beta$. Early in disease, TTR may be upregulated in response to loss of $A\beta$ homeostasis, but as the disease progresses, TTR levels decrease as the protein is trapped in amyloid deposits or as cell death leads to a reduced level of synthesis.

TTR is a homotetrameric soluble protein (55 kDa) that is synthesized in both the liver and the choroid plexus and circulates in blood (3–7 μ M) and CSF (0.1–0.4 μ M).^{28,29} The protein consists of four identical 127-residue subunits, each with two four-stranded antiparallel β -sheets and a short α -helix (Figure 1).^{30,31} Extensive hydrogen bonding between β -strands H and F of two monomers forms the dimer. The packing of two dimers produces a

hydrophobic channel in which the thyroid hormone thyroxine (T4) coordinates to residues in the inner β -sheet.³² Two T4 molecules may bind per TTR tetramer. The first T4 binds with high affinity ($K_d \sim 10$ nM) to TTR, but the second has lower affinity due to negative cooperativity; the TTR-T4 complex predominantly exists with a 1:1 stoichiometry *in vivo*.³³ TTR serves as the primary carrier of T4 in CSF and as a minor carrier in blood.^{31,34} TTR also facilitates the delivery of retinol from blood to peripheral tissue through its interaction with retinol-binding protein (RBP; 21 kDa; 2–4 μ M).³⁵ Most RBP (~95%) in circulation is complexed with TTR to prevent glomerular filtration and renal catabolism of RBP.³⁶ Holo-RBP (hRBP) coordinates with three subunits of TTR, making contact with the EF helix loop, AB loop, and GH loop in subunits B and C as well as the FG loop in subunit A (Figure 1).^{37,38} Although two hRBPs can bind per TTR tetramer, the predominant species *in vivo* is a 1:1 complex.³⁹ The affinity of hRBP for TTR ($K_d \sim 0.20$ – 0.35 μ M)^{37,40,41} decreases upon loss of retinol.⁴⁰ In human serum, approximately half of TTR is complexed with hRBP and 10–20% with T4. The RBP-binding sites on TTR are orthogonally positioned to the nonoverlapping thyroxine-binding sites;^{32,37,38} no competition has been observed.⁴² RBP is also synthesized in the choroid plexus and circulates in CSF (6–12 nM), where it likely plays a role in retinol uptake and transport in the brain.^{43,44}

Multiple investigators have shown that TTR binds directly to A β and inhibits A β aggregation.^{17–19,45,46} Furthermore, TTR-mediated inhibition of A β toxicity is directly linked to its inhibition of aggregation.^{19,47} We and others have shown that binding of A β to TTR involves residues in β -strands G and H in the “inner” β -sheet of TTR (Figure 1) in or near the thyroxine-binding sites.^{46–48} There is some evidence supporting the existence of a weak secondary interaction between A β and TTR’s solvent-exposed EF α -helix (Figure 1).^{19,47,49} TTR binds significantly more A β oligomers than A β monomers,^{19,50} a factor that can explain how TTR is effective at inhibiting aggregation and toxicity at well below stoichiometric TTR:A β ratios.

TTR’s efficacy at binding A β , inhibiting aggregation, and/or inhibiting toxicity is inversely correlated with TTR tetramer stability.^{46,47,50,51} For example, A β binding, inhibition of aggregation, or inhibition of toxicity was strongest with an engineered TTR mutant that is a stable monomer (mTTR), moderate for wild-type (wt) human TTR (huTTR), and weakest for wt murine TTR (muTTR), which forms more stable tetramers than huTTR does.^{46,47,50} Intriguingly, Li et al. reported a modest ($K_d \sim 20$ μ M) affinity of A β monomers for huTTR but could detect no evidence of monomeric A β binding to mTTR,⁴⁶ a result supported by a more recent investigation.⁵²

The putative A β -binding sites on TTR overlap with those of the natural ligands, T4 and RBP. T4 and RBP stabilize the TTR tetramer against dissociation at acidic pH,^{42,53} and RBP stabilizes TTR tetramers against dissociation or subunit exchange under physiological conditions.^{54,55} To date, *in vitro* investigations of TTR—A β interactions have been conducted in the absence of these ligands. In this study, we examined whether either ligand interferes with or alters TTR’s ability to protect against A β aggregation.

MATERIALS AND METHODS

Transthyretin Production, Purification, and Modification.

Wild-type human recombinant transthyretin (TTR) was produced and purified as previously described.^{47,51,56} An extinction coefficient of $77600 \text{ M}^{-1} \text{ cm}^{-1}$ at 280 nm and a molecular weight of 55044 Da were used to determine the TTR tetramer concentration.⁵⁷

Transthyretin was fluorescently labeled with AlexaFluor 594 (AF594) C₅ Maleimide (ThermoFisher) for FRET binding assays. The conjugation scheme followed a maleimide reaction to TTR's solvent-exposed cysteine residue (maximum of one per TTR monomer). AF594 was dissolved in anhydrous *N,N*-dimethylformamide (DMF, Sigma) to a concentration of 2 mM and then diluted into TTR (6 μM) in PBSA [10 mM Na₂HPO₄/NaH₂PO₄, 150 mM NaCl, and 0.02% (w/v) NaN₃ (pH 7.4)] to a final concentration of 200 μM [8% (v/v) DMF]. The TTR/AF594 mixture vial was wrapped in aluminum foil and gently shaken for 2 h at room temperature for dye conjugation. The mixture was dialyzed against PBSA for 48 h at 4°C to remove excess unconjugated AF594. The TTR tetramer conjugation efficiency was 65%. The protein concentration was determined from the absorbance at 280 and 588 nm using extinction coefficients of 77600 and 96000 $\text{M}^{-1} \text{ cm}^{-1}$ (Thermo Fisher) for TTR and AF594, respectively. A correction factor of 0.51 was used to account for the 280 nm absorbance contribution of AF594. TTR—AF594 conjugates were concentrated to 24 μM in PBSA and stored at 4 °C wrapped in foil.

Preparation of the Transthyretin—Ligand Complex.

A purified native human plasma apo-retinol-binding protein 4 (Fitzgerald Industries International, Acton, MA) stock was diluted to 30 μM in PBSA. The apo-RBP (aRBP) concentration was measured by the absorbance at 280 nm using an extinction coefficient of $40400 \text{ M}^{-1} \text{ cm}^{-1}$ and a molecular weight of 21000 Da.⁵⁸ Synthetic all-*trans*-retinol (Sigma-Aldrich) was dissolved in ethanol at a concentration of approximately 3 mM, and the concentration was determined by the absorbance at 325 nm using an extinction coefficient of $52480 \text{ M}^{-1} \text{ cm}^{-1}$ (Sigma-Aldrich). Holo-RBP (hRBP, 12 μM) was prepared by diluting retinol into aRBP in PBSA to final concentrations of 12 μM aRBP and 15 μM retinol (1:1.25 molar ratio); the mixture was incubated for 4 h at RT in the dark (final concentration of ethanol of <0.3%). The absorbance at 330 nm confirmed retinol binding (data not shown). The A_{330}/A_{280} ratio of hRBP was approximately 0.80, which corresponds to 85% saturation of RBP with retinol.⁵⁹ Retinol binds aRBP with a 1:1 stoichiometry and an affinity of 50–80 nM.⁶⁰ RBP samples were filtered (0.22 μm) before being used. All retinol-containing samples were shielded from light, and fresh batches were regularly produced to minimize retinol oxidation. L-Thyroxine (T4, Sigma-Aldrich) was dissolved in 0.22 μm filtered 15 mM NaOH; the concentration was measured using an extinction coefficient of $6180 \text{ M}^{-1} \text{ cm}^{-1}$ at 325 nm.⁶¹

Transthyretin—ligand complexes (6 μM) were prepared by diluting aRBP, hRBP, or T4 into TTR at a 1:1 molar ratio and incubating the mixture at room temperature overnight in the dark. Binding of retinol to aRBP and hRBP to TTR was confirmed by FRET (Figure S1). The fluorescence anisotropy (monitoring 460 nm emission at 330 nm excitation) of hRBP

and that of TTR with hRBP (1:1) were 0.19 ± 0.02 and 0.26 ± 0.02 , respectively, further confirming binding of hRBP to TTR.⁴¹ After preparation, all samples were stored at 4 °C wrapped in foil.

β -Amyloid Preparation.

Lyophilized A β (1—40) (A β 40, American Peptide) was dissolved in 0.22 μ M filtered 50% acetonitrile to a concentration of 1 mg/mL, frozen at -80 °C, and rethawed. A β 40 was redissolved in filtered (0.22 μ M) denaturing buffer [8 M urea and 100 mM glycine-NaOH buffer (pH 10)] to a final concentration of 2.8 mM. A β 40 aliquots of 4 or 8 μ L were snap-frozen and stored at -80 °C. To further disaggregate A β 40 immediately before each experiment, aliquots were thawed and incubated with a final concentration of 80 mM NaOH for 15 min at RT. A β 40 was diluted into 0.22 μ M filtered PBS [10 mM Na₂HPO₄/NaH₂PO₄ and 150 mM NaCl (pH 7.4)] or PBSA to initiate aggregation. A β 40 monomers have a molecular weight of 4330 Da and an extinction coefficient of 1490 M⁻¹ cm⁻¹ at 280 nm.⁶² All A β 40 concentrations in this study are reported in terms of equivalent monomer molar concentrations. To prepare A β 40 oligomers, A β 40 monomers (80 μ M) in PBSA were incubated at RT for 24 h and diluted to experimental concentrations prior to use.⁶³

HiLyte Fluor 488-labeled A β 40 (A β -488, Anaspec) was reconstituted in 0.22 μ M filtered denaturing buffer to a final concentration of 1.6 mM. Small volumes were aliquoted, snap-frozen, and stored at -80 °C. Oligomeric A β 40 with fluorescent tracers was produced by diluting an aliquot of A β -488 into a stock of unlabeled A β 40 monomers in PBSA at a 1:36 molar ratio of A β -488 to unlabeled A β 40 and then incubating at RT for 24 h wrapped in foil. A β -488 was used only in FRET binding assays with TTR-594.

Thioflavin T Fluorescence.

Thioflavin T (ThT; Fisher Scientific, Fair Lawn, NJ) at 11 μ M in PBSA (0.22 μ M filtered) was prepared using an extinction coefficient of 26.6 mM⁻¹ cm⁻¹ (416 nm in ethanol).⁶⁴ A β 40 (28 μ M) was incubated alone or with TTR, aRBP, hRBP, T4, TTR aRBP, TTR+hRBP, or TTR +T4 (3.6 μ M) in PBSA at 37 °C for 1 or 48 h. A β 40 (28 μ M) was also incubated with 0.5, 1.0, or 1.8 μ M TTR in PBSA at 37 °C for 1 or 48 h. Immediately prior to each measurement on a QuantaMaster spectrofluorometer, 10 μ L of protein was mixed with 130 μ L of 11 μ M ThT and excited at 440 nm with emission recorded from 455 to 500 nm. Retinol does not absorb between 440 and 500 nm.⁴¹ The mean and standard deviation of triplicate measurements at 480 nm minus the background of ThT alone in PBSA were used to estimate the relative mass content of A β fibrils in each sample. Experiments were repeated multiple times with similar results. In separate experiments, A β 40 (28 μ M) was incubated alone or with TTR or TTR+hRBP (3.6 μ M) in PBSA at 37 °C for 60 h; aliquots were removed at several time points, and the ThT fluorescence intensity was measured as described. The kinetic data were fitted to a logistic sigmoidal growth equation to obtain parameters a , k_{app} , and t_{50} , which represent the maximum relative ThT signal, the apparent rate constant of A β fibril growth, and the time to reach 50% of the maximum relative ThT signal, respectively.^{65–67}

Dynamic Light Scattering.

A β 40 (28 μ M final concentration) was diluted into TTR, hRBP, or TTR+hRBP (3.6 μ M final concentration) in filtered (0.02 μ m) PBSA and then immediately filtered (0.22 μ m) directly into an extensively cleaned light scattering cuvette and positioned in a bath of index-matching solvent decahydronaphthalene temperature controlled at approximately 37 °C. Using a Brookhaven BI-200SM system (Brookhaven Instruments Corp., Holtsville, NY) and an Innova 90C-5 argon laser (Coherent, Santa Clara, CA) operating at 488 nm and 150 mW, light scattering data were collected at 90°. The *z*-averaged hydrodynamic diameter ($\langle d_h \rangle_z$) for each sample was calculated from the autocorrelation function using the method of cumulants.⁶⁸ $\langle d_h \rangle_z$ includes contributions from all species in solution (A β 40 monomer, A β 40 aggregates, TTR, hRBP, or TTR—hRBP complexes) but is weighted more heavily toward the larger species. DLS kinetic data were fitted to a power-law equation to determine the rate of A β 40 aggregate size growth (see the Supporting Information).

Nanoparticle Tracking Analysis.

Nanoparticle tracking analysis (NTA) measurements were collected using a Nanosight LM10 instrument (Nanosight, Amesbury, U.K.) equipped with a 405 nm laser. A β 40 (28 μ M) alone or with TTR, hRBP, or TTR+hRBP (3.6 μ M) in PBSA was filtered (0.02 μ m) and injected into the sample chamber using a syringe. Experiments were conducted at RT (~20 °C). One 90 s video was captured at multiple time points between 0 and 2 h after sample preparation with the camera level set to the maximal value. The experiment was stopped when the number of particles reached the upper limit recommended by the instrument manufacturer. The data were recorded and analyzed using NTA version 2.3. TTR, hRBP, or buffer (PBSA) alone contained negligible particle counts (data not shown).

Enzyme-Linked Immunosorbent Assay (ELISA).

Protein stocks were prepared at concentrations of 5—10 μ M and diluted immediately prior to use. ELISA plates (Corning, Inc., Corning, NY) were coated with 50 nM TTR, hRBP, or TTR+hRBP (100 μ L/well) in coating buffer [10 mM sodium carbonate, 30 mM sodium bicarbonate, and 0.05% NaN₃ (pH 9.6)] overnight at 4 °C protected from light. The plate was washed at least three times with wash buffer (200 μ L per well per wash; PBS with 0.05% Tween 20) and incubated with protein-free blocking buffer (300 μ L/well; Pierce, Rockford, IL) for 2 h at RT. As negative controls, TTR+hRBP were not coated but wells were still blocked. A β 40 (86 μ M in PBS) was incubated at RT for 1 day to form oligomers, then diluted to 250, 400, 600, or 750 nM in PBS, and immediately added to protein-coated or negative control wells (50 μ L/well). The plate was incubated at 37 °C for 1 h. After washing, anti-A β antibody 6E10 (Covance, Princeton, NJ) in wash buffer (1:3000) was added to each well (100 μ L/ well) and incubated at RT for 1 h with gentle shaking. After washing, the anti-mouse HRP antibody (1:3000 dilution; Pierce) was added to each well (100 μ L/well) and incubated for 1 h at RT with gentle shaking. After several washes, 100 μ L of a 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (Pierce) was added to each well. Color development (15—30 min incubation at RT) was stopped by addition of 100 μ L of 2 M sulfuric acid per well. The absorbance was measured at 450 nm with an EL800 Universal Microplate Reader (Biotek Instruments, Inc., Winooski, VT). A β 40 binding was

calculated as the mean of four replicate wells minus the mean of the negative control ($A\beta$ 40 without TTR, hRBP, or TTR and hRBP) absorbance.

Experiments measuring binding of aRBP and hRBP (50 nM) to $A\beta$ 40 monomers, oligomers, and fibrils were conducted similarly as described above, except for the preparation of $A\beta$. $A\beta$ 40 (80 μ M) was freshly prepared (monomers) or incubated at RT for 24 h to form oligomers and at 37 °C for 24 h to form fibrils.⁶³ Prior to being added to wells, $A\beta$ 40 was diluted to a concentration of 200 nM in PBS. $A\beta$ 40 binding was calculated as the mean of three replicate wells minus the mean of the negative control ($A\beta$ 40 monomers, oligomers, or fibrils without aRBP or hRBP) absorbance.

Steady State Fluorescence Resonance Energy Transfer.

All measurements were taken using a QuantaMaster spectrofluorometer. $A\beta$ 40 oligomers binding fluorescently tagged TTR followed the FRET scheme illustrated in panels A and B of Figure S2. $A\beta$ 40 oligomers (28 μ M) with fluorescent tracers (36:1 molar ratio of unlabeled $A\beta$ 40 to $A\beta$ -488) were incubated with varying concentrations of TTR-594 with or without hRBP (0, 100, 250, 500, 1000, 1500, 2000, 3500, and 5000 nM) in PBSA for 2 h at 37 °C wrapped in foil. Fluorescence spectra of all samples were measured using an excitation of 504 nm and emission scan from 525 to 700 nm. Background fluorescence spectra of TTR-594 with or without hRBP in PBSA at each concentration were subtracted using an excitation of 504 nm. The emission of the donor ($A\beta$ -488) at 544 nm was used to determine the FRET efficiency (E):

$$E = 1 - \frac{F_{DA}}{F_D} \quad (1)$$

where F_{DA} and F_D are the emissions at 544 nm in the presence and absence of the acceptor (TTR-594), respectively. The mean and standard deviation (triplicate experiments) of FRET efficiency as a function of acceptor concentration were reported. Affinities (K_d) for binding of $A\beta$ -488 oligomers to TTR-594 tetramers were determined by fitting to a 1:1 binding model (see the Supporting Information).⁶⁹ Binding of TTR-594 and hRBP was confirmed by an increase in fluorescence anisotropy compared to that of hRBP alone (data not shown).

FRET of intrinsic aromatic residues of hRBP and/or TTR (donor) to bound retinol (acceptor) followed the schemes illustrated in panels C—F of Figure S. $A\beta$ 40 monomers (80 μ M in PBSA) were incubated for 24 h at RT to form $A\beta$ 40 oligomers, then diluted in PBSA (5 or 15 μ M $A\beta$ 40) alone or with either hRBP or TTR and hRBP (1 μ M), and incubated at 37 °C for 1 h. FRET was monitored by measuring the fluorescence spectra using an excitation of 280 nm and emission scan from 295 to 550 nm. The retinol fluorescence was monitored using an excitation of 330 nm and emission scan from 350 to 550 nm. The mean of triplicate spectra minus the background (PBSA or $A\beta$ 40 alone) was reported. Unbound, free retinol exhibits negligible fluorescence in PBSA; the fluorophore alone is quenched in aqueous solvents.

Gel Electrophoresis (SDS—PAGE) of TTR—hRBP—A β Complexes.

For monitoring A β -induced TTR tetramer destabilization, TTR or TTR with hRBP (3.6 μ M) was mixed with A β 40 monomers (60 μ M) in PBSA and then incubated for 0–50 h at 37 °C to induce A β 40 aggregation. Samples were diluted into Tris-glycine SDS sample buffer (Novex) and briefly centrifuged. Control TTR and TTR/hRBP samples were boiled for 10 min to use as RBP and monomeric TTR standards on the gel. Samples were loaded in a 10 to 20% polyacrylamide gradient gel (Novex), electrophoresed in Tris-glycine SDS running buffer (Novex), and stained with Coomassie blue. Destained gels were photocopied, and the densitometry of each protein bands was quantified using ImageJ. The percentage of total TTR tetramer dissociation was determined by the density ratio between the amount of monomeric TTR and total TTR protein. It was reported as the mean and standard deviation of three replicate gel assay experiments.

RESULTS

hRBP Interferes with TTR-Mediated Inhibition of A β Aggregation.

We and others have previously demonstrated that TTR inhibits A β aggregation and toxicity in vitro.^{16,18,19,46,47,50,70} These experiments were conducted in the absence of TTR's natural ligands, hRBP and T4. RBP's binding to TTR involves residues in the EF α -helix' overlapping with a possible A β -binding domain (Figure 1).^{37,38,47} T4 binds in the hydrophobic cavity of the TTR tetramer, interacting with several residues known to be important for A β -TTR binding.^{32,47} We therefore inquired if either RBP or T4 would affect the protective ability of TTR against A β aggregation.

The A β 40 fibril content was monitored by ThT, a fluorescent dye that is widely used as a measure of the mass of amyloid fibrils.^{71,72} A β 40 alone was initially ThT-negative but developed a strong ThT-positive signal by 48 h, as expected (Figure 2A). TTR (7:1 A β 40:TTR molar ratio) decreased the ThT intensity to 25% of that of A β 40 alone at 48 h, consistent with previously published data.⁵¹ In contrast, a 1:1 mixture of TTR and hRBP decreased the ThT intensity to only 60% of that of A β 40 alone at 48 h, indicating that TTR with hRBP is much less effective at inhibiting A β 40 fibrillation than TTR alone ($p < 0.001$). On the other hand, a 1:1 TTR and aRBP mixture was nearly as effective at inhibiting A β 40 fibrillation as TTR alone.

Given an estimate for K_d of 0.35 for binding of hRBP to TTR at a 1:1 stoichiometry,³⁷ free hRBP and TTR will be in equilibrium with the TTR-hRBP complex and must be accounted for. We calculated that 27% of TTR (1 μ M) is not bound to hRBP at equilibrium under our experimental conditions. At 1 μ M, TTR decreased the ThT intensity to 60% of that of A β 40 alone (Figure 2B), similar to the result with the 3.6 μ M TTR+hRBP mixture (Figure 2A). aRBP binds more weakly to TTR than hRBP does ($K_d \sim 1.20 \mu$ M).⁴⁰ Under our experimental conditions, the 1:1 TTR+aRBP mixture would contain 43% unbound TTR ($\sim 1.6 \mu$ M) at equilibrium. For comparison, TTR alone at a similar concentration (1.8 μ M) (Figure 2b) was not as effective as the 3.6 μ M TTR+aRBP mixture at inhibiting A β 40 fibrillation (Figure 2A). This suggests that, unlike the case for hRBP, when aRBP is complexed to TTR, it does not affect TTR's ability to hinder A β fibrillogenesis. Taken

together, these data indicate that the decrease in ThT intensity with the TTR+hRBP mixture is likely due to the inhibitory activity of uncomplexed TTR and that the TTR-hRBP complex is ineffective at inhibiting A β fibrillation.

TTR with bound thyroxine was also less effective against A β 40 fibrillation than uncomplexed TTR was (Figure 2A). However, because the population of uncomplexed TTR is very low given the strong binding affinity ($K_d = 10$ nM),³³ we conclude that the TTR-T4 complex still exhibits inhibitory activity against A β 40 fibrillation, albeit modestly less than free TTR. Because the effect of hRBP was much stronger, we chose to focus our remaining work on hRBP.

Next, ThT was used to measure fibrillogenesis kinetics for A β 40 alone or co-incubated with TTR or TTR+hRBP (Figure 3). The data were fit to a simple sigmoidal growth curve (eq 2),

$$y = \frac{a}{1 + \exp[-k_{\text{app}}(t - t_{50})]} \quad (2)$$

where y is the ThT signal at time t , a is the maximum ThT signal (normalized to A β 40 alone), k_{app} is the apparent rate constant of fibril growth, and t_{50} is the time to reach 50% of the maximum signal. For all three curves, there was a lag of ~5 h before observation of the ThT-positive signal. A β alone reached a plateau at 48 h. Upon co-incubation with TTR and hRBP, fibrillation was slower, but at long times, the ThT signal approached 70% of that of A β alone. In contrast, co-incubation with TTR resulted in a ThT signal that plateaued at only 20% of that of A β , indicating that TTR partially prevents, not simply delays, A β fibrillogenesis. k_{app} was lower when A β 40 was incubated with TTR compared to A β 40 alone ($p < 0.05$). For A β 40 with TTR and hRBP, k_{app} was intermediate between those of A β 40 alone and A β 40 with TTR (Table S1). Values of t_{50} for the three samples ranged from 19 to 27 h but were not statistically different (Table S1).

Further confirmation that hRBP impairs TTR's efficacy as an anti-aggregation agent was sought from light scattering experiments. The size of A β aggregates and the rate of growth were monitored by dynamic light scattering (DLS). While ThT fluorescence reports on the mass of amyloid fibrils, DLS provides information about the size of particles in solution. The technique is particularly useful at detecting aggregation at early times, prior to the onset of the ThT signal, because of its high sensitivity to very low levels of aggregates. The mean hydrodynamic diameter ($\langle d_h \rangle_z$) of A β 40 (28 μM) in solution increased rapidly over time (Figure 4). The hydrodynamic diameter at time zero was reduced when either TTR or TTR+hRBP (3.6 μM) were added, because $\langle d_h \rangle_z$ includes both A β 40 aggregates (150 nm) and TTR or TTR and hRBP (6 or 8 nm, respectively). The increase in aggregate size was measured, and the data were fit to a power-law equation

$$\langle d_h \rangle_z = \langle d_h \rangle_{z0} + k_{\text{obs}} t^\alpha \quad (3)$$

where $\langle d_h \rangle_{z0}$ is $\langle d_h \rangle_z$ at time zero, k_{obs} is the observed rate constant for growth, and v provides some indication of the primary mechanism of growth (see the Supporting Information). TTR significantly slowed A β 40 growth (Figure 4), consistent with ThT results (Figure 3) as well as previous reports.^{19,51} In contrast, TTR and hRBP did not slow the growth of A β 40 aggregates, as seen by comparison of k_{obs} values (Table S2).

We further analyzed the effect of TTR+hRBP on A β 40 aggregate size using nanoparticle tracking analysis (NTA), a particle-by-particle scattering technique, in which both the total number of particles and the particle size distribution can be ascertained. Only ≥ 30 nm particles are reliably detected.^{73,74} TTR, hRBP, and TTR–hRBP complexes are smaller than 30 nm and therefore invisible with this technique, as are the A β 40 monomer and smaller oligomers. A β 40 (28 μM) was incubated alone, with 3.6 μM TTR, or with TTR+hRBP for 2 h. Data are reported as the particle number concentration as a function of time (Figure 5A) or as the particle size distribution 10 or 60 min after preparation (panel B or C of Figure 5, respectively). TTR reduced both the number concentration of large A β 40 aggregates (Figure 5A) and the aggregate size (figure 5B,C), indicating that the protein suppressed the formation of new A β aggregate particles as well as the growth of preexisting aggregates. In contrast, the total number of A β 40 aggregate particles was essentially unaffected by TTR+hRBP (Figure 5A). Furthermore, size distributions were shifted toward larger A β 40 aggregates upon incubation with TTR+hRBP as compared to those in their absence (Figure 5B,C). The increase in aggregate size with TTR+hRBP could be explained by hypothesizing that TTR–hRBP complexes bind to A β 40 aggregates but do not inhibit their growth. If the TTR–hRBP complex did not interact with A β 40 at all, one would expect to see the same size distribution for A β 40 alone compared to that with the mixture of A β 40, hRBP, and TTR because TTR–hRBP complexes are too small to be detected by NTA. In all cases, the aggregates that did form were fibrillar, as imaged by TEM (Figure S3).

Because an estimated 27% of hRBP remains dissociated from TTR under our experimental conditions, we tested whether hRBP in the absence of TTR affected A β 40 aggregation. By all measures (ThT, DLS, and NTA), we observed that hRBP modestly suppressed A β 40 aggregation (Figures S4 and S5). Thus, although either hRBP or TTR by itself can mildly (hRBP) or strongly (TTR) inhibit A β 40 aggregation, the TTR–hRBP complex is a poor inhibitor of aggregation.

The Transthyretin–hRBP Complex Binds β -Amyloid.

We next asked why TTR–hRBP complexes were less effective than TTR at inhibiting A β 40 aggregation. TTR must bind to A β to inhibit its aggregation,¹⁹ so direct interference of hRBP with binding of A β to TTR could explain the loss of efficacy. To determine whether hRBP interfered with binding of A β 40 to TTR, we conducted a solution-based fluorescence resonance energy transfer (FRET) assay. Briefly, A β 40 was mixed with HiLyte-488-labeled A β 40 (36:1 A β :A β -488 molar ratio, total A β 40 concentration of 28 μM), incubated at RT for 24 h to form oligomers, and then added to TTR labeled with AlexaFluor 594 (TTR-594) or TTR-594 and hRBP. Binding was measured by an increase in FRET efficiency as a function of TTR-594 concentration (Figure 6A). The data were fit to a simple 1:1 binding model to obtain estimates of pseudo K_d of 2.6 ± 0.8 μM for A β oligomers binding to TTR

tetramers and $1.9 \pm 0.8 \mu\text{M}$ for A β oligomers binding to TTR and hRBP (Figure S6 and Table S3).⁶⁹ These are pseudo K_d 's because the binding model does not account for the heterogeneous and oligomeric nature of the A β preparation. There was no statistical difference between the two affinities, indicating no effect of hRBP on binding of A β 40 to TTR.

We confirmed this finding by an ELISA. In brief, TTR or TTR with hRBP was immobilized on ELISA plates, A β 40 oligomers were added to each well, and binding was detected by anti-A β antibodies. There was no statistical difference in the amount of A β 40 bound to TTR versus the TTR–hRBP complex (Figure 6B).

As shown in Figure 6B and Figure S7, hRBP alone also bound A β 40, although less than TTR or the TTR–hRBP complex. This is consistent with the weak inhibition of A β 40 aggregation (Figures S4 and S5). We asked next whether A β binds directly to TTR or to hRBP on the TTR–hRBP complex. To do this, we utilized FRET from intrinsic aromatic residues (donor) to bound retinol (acceptor). With hRBP alone, excitation at 280 nm resulted in FRET and retinol emission at ~460 nm. Addition of A β 40 oligomers to hRBP increased the retinol emission intensity (Figure 7A,B and Figure S7B), which we attributed to binding of A β 40 to hRBP (possibly due to additional FRET from A β 40's tyrosine, change in hRBP aromatic residue emission due to A β 40 binding, or enhancement of retinol's quantum yield by further secluding bound retinol from the solvent via A β 40 binding). In contrast, addition of a 15-fold excess of oligomeric A β 40 did not affect the emission of retinol from TTR and hRBP (Figure 7C); no significant change was observed via direct excitation of the acceptor either (Figure 7D). Because binding of A β 40 directly to hRBP caused an increase in retinol emission, we interpreted these data to indicate that, on the TTR–hRBP complex, A β 40 binds to TTR at a site distant from hRBP and not to hRBP. hRBP contains a solvent-accessible hydrophobic surface that is buried upon complexation with TTR, which could explain why A β 40 binds to hRBP differently than to the TTR–hRBP complex.

Taken together, these results demonstrate that oligomeric A β 40 binds equally well to the TTR–hRBP complex and to TTR. Therefore, the loss of TTR-mediated inhibition of A β 40 aggregation by formation of the TTR–hRBP complex cannot be attributed to competition between hRBP and A β 40 oligomers for binding to TTR.

hRBP Stabilizes TTR against A β -Mediated Destabilization.

In previous work, we showed that binding of A β to TTR is required for TTR-mediated inhibition of aggregation, that A β binding appears to destabilize TTR tetramers, and that destabilized TTR mutants are more effective than the wt as inhibitors of A β aggregation.^{19,47,49,51} hRBP is known to stabilize TTR tetramers against dissociation or subunit exchange at physiological pH.^{42,55} Because A β binds to the TTR–hRBP complexes, we postulated that hRBP counteracts A β -mediated destabilization of TTR tetramers and subsequently prevents TTR from inhibiting A β aggregation.

To test this hypothesis, we used an SDS–PAGE assay that we previously developed to observe the stability of TTR tetramers at physiological pH and temperature. Briefly, TTR or the TTR–hRBP complex was mixed with fresh A β 40 and then incubated for 0–20 h at 37 °C

to allow for A β 40 aggregation. These samples along with TTR or the TTR–hRBP complex without A β 40 were diluted into 2% SDS, applied to a gel without boiling, and subjected to electrophoresis. Samples are not cross-linked, so this assay measures the resistance of the protein complex to SDS-induced dissociation. Under these conditions, TTR is stable as a tetramer and does not dissociate into its monomeric subunits,¹⁹ while TTR–hRBP complexes dissociate into their constituent proteins (tetrameric TTR and RBP). To facilitate identification, TTR and the TTR–hRBP complex were boiled and then applied to the same gel. Boiling dissociates TTR into its monomeric subunits (Figure 8A). When TTR was incubated with A β 40, a new band corresponding to the TTR monomer appeared (Figure 8A), as reported previously.¹⁹ By densitometry, we quantified the appearance of the TTR monomer as a function of time. The TTR monomer was detected after co-incubation with A β 40 for ~10 h, with $5 \pm 1\%$ of the total TTR dissociated after 20 h (Figure 8B). The appearance of a measurable amount of TTR monomer corresponded to a decrease in the intensity of the A β 40 monomer/dimer band. This time scale is consistent with the kinetics of A β 40 fibrillogenesis as measured by ThT (Figure 3). This suggests that the association of A β 40 into SDS-resistant, ThT-positive aggregates may trigger TTR destabilization. When A β 40 was co-incubated with TTR and hRBP, no TTR monomer band was detected (Figure 8), showing that hRBP hinders A β -induced dissociation of the TTR tetramer to TTR monomers. Because we have established that A β 40 binds to the TTR–hRBP complex, this result supports the hypothesis that hRBP stabilizes TTR, counteracting A β -induced destabilization.

DISCUSSION

Over the past two decades, evidence that the circulating transport protein TTR may play a neuroprotective role in Alzheimer's disease has accumulated. The earliest reports of interaction between A β and TTR were published more than 20 years ago.^{70,75} The biological implications of this interaction became clearer when studies demonstrated that TTR provides protection against amyloid toxicity in transgenic APP mice.^{9,13,14,18} The relevance of these observations in animal models to human disease has been supported by proteomic studies showing a complex, dynamic relationship between A β and TTR levels in AD.²⁴

These intriguing findings have motivated more detailed investigations of the molecular basis for the interactions between A β and TTR. The primary binding site has been identified as involving residues on TTR's inner β -sheet, facing the interior hydrophobic cavity.^{19,46,47,49} There may be a secondary A β -binding site at or near the EF helix,^{19,47,49} although that finding was not confirmed.⁴⁶

Because the putative A β -binding domains on TTR overlap with binding sites for T4 and hRBP, we examined the effect of these natural ligands on TTR's ability to inhibit A β aggregation. In our initial assessment, we observed that hRBP has a greater adverse impact than T4 on TTR-mediated inhibition of aggregation, so we chose to focus on the role of hRBP. TTR–hRBP complexes are significantly less effective at inhibiting A β 40 aggregation than is TTR, as demonstrated by ThT fluorescence, dynamic light scattering, and nanoparticle tracking analysis (Figures 2–5 and Tables S1 and S2). In addition, any

inhibition of A β 40 aggregation with TTR+hRBP mixtures can be attributed to free TTR (Figure 2). Although by themselves hRBP modestly (Figures S4 and S5) and TTR strongly suppressed A β aggregation, the TTR-hRBP complex is a poor inhibitor of aggregation.

If hRBP blocked binding of A β 40 to TTR, this could explain the loss of TTR-mediated inhibition of A β 40 aggregation. However, A β 40 binds equally well to TTR-hRBP complexes and TTR alone (Figure 6), ruling out competition between A β 40 and hRBP for binding to TTR. Because there are two hRBP-binding sites on TTR but only one is typically occupied (Figure 1), this result does not contradict the possibility of an A β -binding site at or near the EF helix.

To explain the lack of inhibition of aggregation despite the evidence of binding, we considered the fact that hRBP binding is known to stabilize TTR tetramers.^{42,55} If tetramer destabilization is important for inhibition of A β aggregation, then hRBP may prevent this from occurring. We used a gel electrophoresis assay, which detects resistance to SDS as a measure of stability, to test this hypothesis. We observed that A β 40 destabilized TTR tetramers over time and that hRBP prevented this destabilization (Figure 8). Taken together, these data demonstrate that binding of A β and inhibition of its aggregation are separate events: binding of A β to TTR is necessary but not sufficient for inhibition of A β aggregation. Further support for the hypothesis that TTR-hRBP complexes can bind to A β 40 aggregates without inhibiting aggregation comes from NTA experiments, where we observed a measurable shift of the size distribution toward larger aggregates without a change in the number concentration (Figure 5). In addition, aRBP does not stabilize TTR tetramers,⁵⁵ unlike hRBP, and the TTR-aRBP complex is nearly as effective as TTR at inhibiting A β 40 aggregation (Figure 2).

The details of the mechanism of TTR-mediated inhibition of A β aggregation are still a matter of debate. Several groups have established that A β oligomers and fibrils bind to TTR to a greater extent than do A β monomers^{49,50} and that TTR's efficacy at binding A β and inhibiting aggregation is inversely correlated with TTR tetramer stability.^{46,49–51} Li et al.⁴⁶ proposed that the dominant *in vivo* mechanism of TTR-mediated inhibition of A β aggregation was binding of A β monomers, and possibly oligomers, to TTR tetramers, with binding in or near the site occupied by T4. In this model, TTR-A β binding decreases the concentration of A β and thus decreases the rate of initiation of aggregation. We have proposed a somewhat different mechanism. Specifically, our group has hypothesized that binding of A β oligomers to TTR, possibly at the EF α -helix, triggers partial destabilization of TTR tetramers, opening the interior hydrophobic cavity for further A β binding, and that the small fraction of TTR that is naturally monomeric may play an outsized role in sequestering A β oligomers.^{19,47,49,51} In agreement with this, monomeric TTR was shown to be a better inhibitor of curli amyloid formation than is the tetramer.⁷⁶ The data collected in this study support the hypothesis that both binding and tetramer destabilization are needed for inhibition of aggregation.

Both TTR and RBP are synthesized in the choroid plexus and are presumably important for transport of T4 and retinol across the blood-brain and blood-CSF barriers as well as within the CSF and brain interstitial fluids.^{44–77} Using reported concentrations in human CSF of

TTR (0.1–0.4 μM),^{28,29} RBP (6–12 nM),^{43,78} and retinol (6–12 nM),^{43,79} we estimate that only ~2% of CSF TTR is complexed with RBP.⁴⁰ Thus, hRBP-mediated stabilization of TTR is not a factor on the brain side. Furthermore, RBP levels are reportedly lower in AD.^{80,81} Estimates of the concentration of A β in these fluids are typically 1–2 nM in CSF and 100-fold lower in plasma (equivalent monomer molar concentrations).^{46,82–84} Li et al.⁴⁶ reported an estimated K_d of ~20 μM for TTR tetramers binding to A β monomers. Using this value of K_d along with 2 nM A β and 0.2 μM TTR, we calculate that only 1% of the A β in CSF would be bound to TTR. Thus, it is highly unlikely that binding of A β monomers to TTR tetramers could account for any measurable inhibition of A β aggregation under physiologically relevant conditions. Alternatively, several lines of evidence support the notion that A β oligomers bind more than monomers, and in fact, the stable monomeric mutant mTTR reportedly does not bind A β monomers at all^{46,52} yet clearly binds A β oligomers well.^{49,50,52,63} On the basis of estimates of the TTR tetramer/monomer equilibrium, ~5% of TTR in CSF (10 nM) is dissociated to monomers.^{28,29,51} We calculate that TTR monomers are in molar excess over A β in CSF (5-fold excess if A β is a monomer, more so if A β is oligomerized), so even if the concentration of TTR monomers is only a fraction of that of their TTR tetramer counterpart, it is not negligible. The problem is one of affinity rather than of stoichiometry. We estimated a pseudo K_d of 2 μM for a heterogeneous mix of A β 40 monomers and oligomers to TTR tetramers (Figure S6 and Table S3) and argue that the affinity for A β oligomers and TTR monomers would be stronger. K_d values of 10–100 nM would lead to an estimate of 10–50% A β oligomers bound to the TTR monomer. It should be pointed out that TTR has been detected in amyloid plaques in AD,¹⁸ but there is no direct evidence for TTR–A β complexes in CSF. Furthermore, TTR is synthesized and secreted by neurons¹⁸ as well as by the choroid plexus. Thus, the biologically relevant site for TTR-mediated protection against A β could lie in the tissue and brain interstitial fluid as well as or instead of in the CSF.

Our data strongly support the hypothesis that binding of A β to TTR is insufficient for inhibition of aggregation and indicates that destabilization of the TTR tetramer is needed. Furthermore, it is A β binding that appears to trigger TTR destabilization. Not yet known is exactly how destabilization works to enhance inhibition of A β aggregation. One possibility is that TTR monomeric subunits are released, which then bind to A β oligomers. Given the slow release of the monomer in the gel assay (Figure 8) and the close correlation in time between the onset of A β fibril formation (Figure 3) and that of tetramer destabilization (Figure 8), this possibility is unlikely. A second possibility is that the tetrameric structure is simply “loosened” by binding of A β oligomers, weakening steric constraints on access of A β oligomers to the interior cavity. A third is that, by increasing the rate of TTR monomer subunit exchange, A β could more readily compete with TTR reassembly.⁴⁹ Because TTR by itself can misfold into amyloid fibrils and TTR destabilization is an early step in its amyloidogenesis pathway,⁸⁵ any TTR-based therapeutic intervention in AD should not proceed via destabilization of TTR other than when it is directly coupled to A β binding. A strategy based on developing stable mimics of the TTR monomer is more likely to succeed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Aβ	β -amyloid
Aβ-488	HiLyte Fluor 488-labeled A β (1—40)
AD	Alzheimer's disease
AF594	AlexaFluor 594 C ₅ maleimide
APP	amyloid precursor protein
aRBP	apo-retinol-binding protein
CSF	cerebrospinal fluid
DLS	dynamic light scattering
DMF	<i>N,N</i> -dimethylformamide
ELISA	enzyme-linked immunosorbent assay
FRET	fluorescence resonance energy transfer
hRBP	holo-retinol-binding protein
mTTR	engineered double-mutant monomeric transthyretin
NTA	nanoparticle tracking analysis
PBS	phosphate-buffered saline
PBSA	phosphate- buffered saline with azide
RBP	retinol-binding protein
RT	room temperature
SD	standard deviation
SDS—PAGE	sodium dodecyl sulfate—polyacrylamide gel electrophoresis
T4	thyroxine
TEM	transmission electron microscopy
ThT	thioflavin T

TMB	3,3',5,5'-tetramethylbenzidine
TS-FRET	time-resolved fluorescence resonance energy transfer
TTR	transthyretin
TTR-594	TTR tetramer conjugated with AlexaFluor 594
wt	wild type

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Figure 1. Ribbon structure of 1:1 binding of human serum hRBP to human TTR. The TTR tetramer (light gray, with subunits A—D) and hRBP (dark gray) coordinate by predominantly hydrophobic interactions. Residues on TTR (blue) and hRBP (orange) involved in binding are highlighted. Retinol (green) is buried in the hydrophobic cavity of RBP's β -barrel with the alcohol moiety pointing toward the EF loop of TTR. Thyroxine binds in the central cavity (not shown). The putative weak A β -binding domain on the EF α -helix and the strong binding domain on β -strand G on each TTR monomeric subunit are colored red. Generated from Protein Data Bank entry 3BSZ.

Figure 2.

TTR-mediated inhibition of Aβ fibril formation after 48 h. (A) TTR alone (3.6 μM) or complexed with aRBP, hRBP, or T4 (3.6 μM) was incubated with Aβ₄₀ (28 μM) at 37 °C for 1 or 48 h. (B) Aβ₄₀ (28 μM) alone or with TTR (3.6, 1.8, 1.0, or 0.5 μM) was incubated at 37 °C for 1 or 48 h. The Aβ₄₀ fibril content was detected by ThT fluorescence. Data shown are the mean ± SD of three replicates. TTR, aRBP, hRBP, and T4 did not exhibit ThT fluorescence (data not shown). An asterisk indicates the value differs from the value for 48 h Aβ alone ($p < 0.001$). θ indicates the value differs from the value for 48 h TTR (3.6 μM) and

$A\beta$ ($p < 0.001$). w indicates the value differs from the value for 48 h $A\beta$ alone ($p < 0.01$). A number sign indicates the value differs from the value for 48 h TTR ($3.6 \mu\text{M}$) and $A\beta$ ($p < 0.005$).

Figure 3. TTR-mediated inhibition of A β 40 fibril growth kinetics. A β 40 (28 μ M, $\blacklozenge$) alone or with TTR (3.6 μ M, $\blacklozenge$) or TTR+hRBP (3.6 μ M, $\blacksquare$) was incubated at 37 °C for 0–60 h. A β 40 fibril growth was monitored by ThT fluorescence. Data shown are the means $\pm$ SD of three replicates. The curves show the fit to a sigmoidal growth equation (see the Supporting Information).^{65–67}

Figure 4. Effect of TTR or TTR with hRBP on A β 40 aggregate growth kinetics. The mean hydrodynamic diameter of A β 40 alone (28 μ M, $\blacklozenge$) or A β 40 incubated with 3.6 μ M TTR ($\square$) or TTR+hRBP ($\blacksquare$) at 37 $^{\circ}$ C was monitored by DLS. Curves show fits of data to eq 3 (see the Supporting Information). Data shown are from one representative run. The experiment was repeated two or three times with similar results.

Figure 5.

Total aggregate number concentrations and particle size distributions measured by NTA. (A) Aβ40 alone (28 μM, ♦) or with 3.6 μM TTR (□) or TTR+hRBP (■) was incubated at RT. The total Aβ40 aggregate number concentration was reported as a function of time. (B and C) Particle size distributions of Aβ40 aggregates alone (black) or incubated with TTR (gray) or TTR+hRBP (white) at RT for 10 and 60 min, respectively. Data shown are from one representative run. The experiment was repeated three times with similar results.

Figure 6.

Binding of Aβ to TTR and the TTR-hRBP complex. (A) Oligomeric Aβ₄₀ with fluorescent tracers (HiLyte-488) at 28 μM (36:1 Aβ:Aβ-488 molar ratio) were incubated with increasing amounts of TTR-594 without (□) and with hRBP bound (■). The FRET efficiency was measured as a function of FRET acceptor concentration (TTR-594). Data shown are the mean ± SD of three replicates. (B) TTR (□), hRBP (○), and TTR+hRBP (■) were adsorbed to a 96-well plate. Oligomeric Aβ₄₀ (0, 250, 400, 600, or 700 nM) was added to each well and allowed to bind for 1 h. Unbound Aβ₄₀ was removed by washing, and the

amount of A β 40 bound was measured by an ELISA. Data shown are the mean $\pm$ SD of four replicates. These experiments were repeated multiple times with similar results.

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Figure 7. Fluorescence emission spectra of hRBP and the TTR–hRBP complex with A β 40 oligomers. Emission was recorded with excitation at (A and C) 280 nm or (B and D) 330 nm. (A and B) hRBP (1 μ M) was incubated alone or with oligomeric A β 40 (5 or 15 μ M). (C and D) TTR +hRBP (1 μ M) were incubated alone or with oligomeric A β 40 (15 μ M). Data shown are the mean of three replicate spectra minus the background spectra of buffer or A β 40 alone.

Figure 8.

Effect of hRBP on A β -mediated TTR tetramer destabilization. TTR or TTR with hRBP (3.6 μ M) was mixed with A β 40 monomers (60 μ M) and then incubated for 0, 5, 10, or 20 h at 37 °C to induce A β 40 aggregation. (A) SDS-PAGE gel of TTR or TTR with hRBP without (—) or with (+) A β 40. For comparison, TTR and TTR with hRBP without A β 40 was incubated at 37 °C for 20h. TTR and TTR with RBP were also boiled, providing molecular weight markers for hRBP and TTR monomers. (B) Percent of A β -induced TTR tetramer dissociation in the absence (□) and presence of hRBP (■) determined by densitometric measurement of the ratio between the amount of monomeric TTR and total TTR protein. The results shown represent the mean $\pm$ SD of three independent experiments. With longer co-incubation periods with A β 40, the TTR tetramer bands are shifted toward slightly lower molecular weights (Figure S8).