Human prions and plasma lipoproteins

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Prions are composed solely of an alternatively folded isoform of the prion protein (PrP), designated PrP^{Sc}. The polyoxometalate phosphotungstic acid has been used to separate PrP^{Sc} from its precursor PrP^{C} by selective precipitation; notably, native PrP^{Sc} has not been solubilized by using nondenaturing detergents. Because of the similarities between PrP^{Sc} and lipoproteins with respect to hydrophobicity and formation of phosphotungstic acid complexes, we asked whether these molecules are bound to each other in blood. Here we report that prions from the brains of patients with sporadic Creutzfeldt–Jakob disease (CJD) bind to very low-density (VLDL) and low-density (LDL) lipoproteins but not to high-density lipoproteins (HDL) or other plasma components, as demonstrated both by affinity assay and electron microscopy. Immunoassays demonstrated that apolipoprotein B (apoB), which is the major protein component of VLDL and LDL, bound PrP^{Sc} through a highly cooperative process. Approximately 50% of the PrP^{Sc} bound to LDL particles was released after exposure to 4 M guanidine hydrochloride at 80°C for 20 min. The apparent binding constants of native human (Hu) PrP^{Sc} or denatured recombinant HuPrP(90–231) for apoB and LDL ranged from 28 to 212 pM. Whether detection of PrP^{Sc} in VLDL and LDL particles can be adapted into an antemortem diagnostic test for prions in the blood of humans, livestock, and free-ranging cervids remains to be determined.

prion protein | apolipoprotein B | blood | Creutzfeldt–Jakob disease

Prions are composed solely of an alternatively folded isoform of the prion protein, designated PrP^{Sc}. Despite numerous efforts to solubilize native PrP^{Sc}, no procedure has been developed except for incorporation of the protein into liposomes (1, 2). The hydrophobic properties of PrP^{Sc} not only have impeded biological investigations but also have prevented structural studies at atomic resolution.

During the development of an immunoassay for PrP^{Sc} that does not depend upon limited proteolysis to hydrolyze the precursor protein designated PrP^{C}, we found that the Na salt of phosphotungstic acid (PTA) selectively complexes with PrP^{Sc} (3). PTA is a water-soluble salt featuring the nearly spherical trianion $[PW_{12}O_{14}]^{3-}$ that belongs to a broad class of polynuclear transition metal-oxo complexes known as polyoxometalates (POMs; see ref. 4). Recently, two of us (J.G.S. and S.B.P.) investigated the mechanism of selective complex formation between Keggin-type POMs and PrP^{Sc} using a series of POM analogues (5). The ability of POMs to complex with PrP^{Sc} as well as lipoproteins (6) raised the possibility that these two hydrophobic proteins might copurify.

We began by examining the distribution of human (Hu) PrP^{Sc} in human plasma fractions spiked with sporadic Creutzfeldt–Jakob disease (sCJD) prions from human brain. CJD prions were found with very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) but not with high-density lipoproteins (HDL) or any other plasma component. In addition to binding to purified VLDL and LDL particles, PrP^{Sc} was also found to bind apolipoprotein B (apoB), which is the major protein component of both VLDL and LDL. The positive cooperativity of the binding and high avidity of apoB, VLDL, and LDL for native HuPrP^{Sc} determined in capture-affinity assay raised the possibility that these lipoprotein particles may feature in the pathogenesis of prion diseases. Whether our findings will form the basis of an antemortem diagnostic test for prions remains to be established.

Results

To determine the distribution of PrP^{Sc} in four different plasma fractions, 15% (wt/vol) brain homogenates in 4% Sarkosyl from sCJD patients and controls were diluted 100-fold into pooled normal plasma (Fig. 5, which is published as supporting information on the PNAS web site). The protease-resistant core of PrP^{Sc}, denoted PrP 27–30, partitioned into the VLDL and LDL particles, whereas traces were found in the immunoglobulins but none in HDL or albumin fraction.

High-Affinity Binding of sCJD Prions to LDL. To investigate the interaction of PrP^{Sc} with plasma VLDL or LDL, we measured the capture of native PrP^{Sc} by purified LDL or HDL using the CDI format. We coated plates with serial dilutions of purified lipoproteins and then exposed them to PrP^{Sc} precipitated with PTA from sCJD brain homogenate. After washing the plates to remove unbound PrP^{Sc}, we denatured the PrP^{Sc} for it to bind to the europium (Eu)-labeled mAb 3F4 used for detection. After denaturation of PrP^{Sc}, which was accomplished with 4 M guanidine hydrochloride (Gdn·HCl) at 80°C for 20 min, the Gdn·HCl was removed, and the plates were washed again. We observed that PrP 27–30 in sCJD brain homogenates was captured as a function of the concentration of LDL (Fig. 1*A*) and VLDL (data not shown). Despite a similar lipid composition (6), HDL did not capture PrP 27–30 (Fig. 1*A*).

We next asked what fraction of PrP^{Sc} was released from LDL upon denaturation. As shown in Fig. 1*B*, measurable PrP^{Sc} was released upon denaturation. LDL-coated plates were exposed to PrP^{Sc} from sCJD brain homogenates that had been precipitated with PTA and then denatured by using Gdn·HCl at 80°C for 20 min. The PrP^{Sc} bound to LDL was measured by adding the detection antibody to the plate after the Gdn·HCl was removed (Fig. 1*B*, circles). The unbound PrP^{Sc} in the Gdn·HCl was measured by the CDI after the sample was diluted 10-fold. As shown, the level of unbound PrP^{Sc} is ≈10% of that found for the PrP^{Sc} that remained bound (Fig. 1*B*, triangles). When we take into account that the unbound PrP^{Sc} sample was diluted 10-fold before the CDI to lower the Gdn·HCl concentration below a level that would denature the capture and detection antibodies, the CDI readings are equal to the readings of the original LDL-coated plate (Fig. 1). Therefore, we conclude that ≈50%

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Abbreviations: PTA, phosphotungstic acid; CJD, Creutzfeldt–Jakob disease; sCJD, sporadic CJD; PrP, prion protein; HuPrP, human PrP; LDL, low-density lipoprotein; VLDL, very LDL; HDL, high-density lipoprotein; apoB, apolipoprotein B; apoE, apolipoprotein E; CDI, conformation-dependent immunoassay; Gdn·HCl, guanidine hydrochloride; TRF, time-resolved fluorescence; recHuPrP, recombinant HuPrP.

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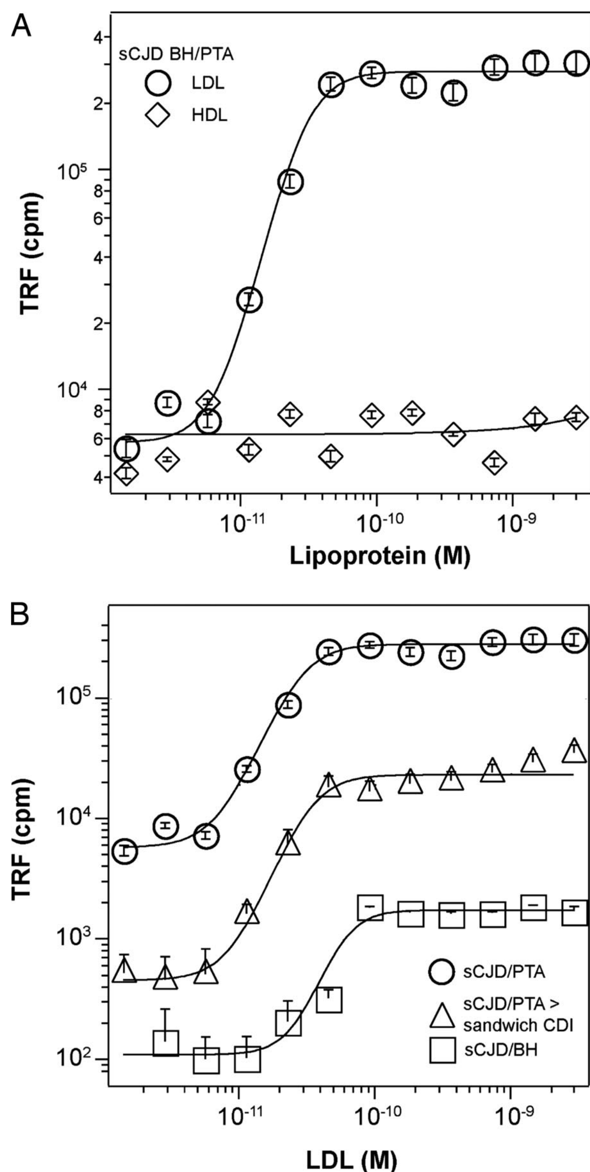

Fig. 1. Native PrP^{Sc} binding to LDL particles. Prions in either sCJD brain homogenates (sCJD/BH) or PTA-precipitated samples (sCJD/PTA) bound to LDL; Gdn·HCl-mediated denaturation released ≈50% of the PrP^{Sc} bound to LDL. (*A*) rPrP^{Sc} in PTA-precipitated samples bound to LDL (circles) but not HDL (diamonds). Lipoprotein-coated plates were exposed to rPrP^{Sc} isolated in 5% sCJD brain homogenates after a 1-h treatment with 10 μg/ml PK in the presence of 4% Sarkosyl followed by precipitation with 0.32% PTA and 2.6 mM $MgCl_2$. (*B*) LDL-coated plates were exposed to 20-fold diluted 5% sCJD brain homogenates in 4% Sarkosyl (sCJD/BH) treated previously with 10 μg/ml PK for 1 h at 37°C or to rPrP^{Sc} precipitated from PK-treated sCJD brain homogenate with 0.32% PTA and 2.6 mM $MgCl_2$ (sCJD/PTA). The rPrP^{Sc} bound to LDL was denatured by 4 M Gdn·HCl for 20 min at 80°C and detected by Eu-labeled 3F4 mAb added to the plate after the Gdn·HCl was removed (circles). The released PrP^{Sc} in the Gdn·HCl was measured by the CDI after the sample was diluted 10-fold (triangles). PrP^{Sc} in sCJD brain homogenates designated sCJD/BH (squares) was bound to LDL-coated plates and measured by adding the detection Eu-labeled 3F4 mAb to the plate after the Gdn·HCl was removed. Samples were diluted (20-fold for sCJD/BH and 2.5-fold for sCJD/PTA) in blocking buffer [TBS, pH 7.8, containing 0.25% (wt/vol) BSA, and 0.1% (wt/vol) Tween 20]; incubated on plates previously coated with serially diluted lipoproteins; and, after Gdn·HCl-mediated denaturation *in situ*, assayed for PrP^{Sc}. The time-resolved fluorescence (TRF) values (average ± SD) are from three experiments and are directly proportional to the concentration of PrP^{Sc}. The unbound PrP^{Sc} in Gdn·HCl was diluted 10-fold before the CDI to lower the Gdn·HCl concentration below a level that denatures the capture antibodies.

of the PrP^{Sc} bound to LDL particles is released by the Gdn·HCl denaturation procedure.

We also examined whether LDL can capture prions directly from sCJD brain homogenate. LDL-coated plates were exposed to PrP 27–30 that was generated from sCJD brain homogenate by limited digestion with proteinase K; the level of PrP 27–30 bound, determined by the direct CDI, was ≈50-fold lower than that in the PTA precipitate (Fig. 1*B*; compare circles and squares). The lower signal for the brain homogenate is largely due to the 20-fold dilution necessary to diminish the concentration of Sarkosyl in the homogenate before PrP^{Sc} binding to LDL. Apparently, Sarkosyl inhibits the interaction between PrP^{Sc} and LDL (data not shown).

Because LDL, HDL, and prions polymerized into amyloid rods are visible by electron microscopy, we performed a series of mixing experiments. We examined the sCJD preparations using immunolabeling and negative-stain electron microscopy. PrP 27–30 in sCJD brain homogenates polymerizes into rod-shaped particles, as reported (Fig. 2*A*; see ref. 7). The sCJD rods appear to be more rigid with more well defined contours than those obtained from prion-infected Syrian hamster brains (3, 8). Upon removal of the PTA by dialysis, the morphology of the human CJD rods remained unchanged. Immunogold labeling with an α-PrP mAb confirmed that the rods contain rPrP^{Sc} (Fig. 2*B*). Negative staining of purified preparations of LDL and HDL revealed the typical sizes and morphologies for both lipoprotein particles (Fig. 2 *C* and *D*).

After mixing PTA-precipitated prion rods from sCJD brain with 0.3 mg/ml LDL or HDL, we observed that almost all LDL particles bound to the rods (Fig. 2*E*). Only ≈10% of LDL particles were observed at distances >50 nm from the prion rods. LDL particles bound to prion rods showed a pronounced propensity to fuse into larger aggregates (Fig. 2*E*). This aggregation was presumably triggered by the extreme hydrophobicity of PrP 27–30 (2, 9). In contrast, HDL particles were found all over the grid surface, without any apparent preference for PrP 27–30 (Fig. 2*F*). More than 95% of HDL particles were at distances >50 nm from the human prion rods, demonstrating that HDL has no perceptible propensity to bind PrP 27–30. HDL particles that were in direct proximity of PrP 27–30 showed no aggregation and retained their typical size and structure. These ultrastructural findings combined with the immunoassay data presented above argue that PrP^{Sc} binds to LDL but not to HDL particles.

To estimate the parameters of the binding interaction, the series of isotherms from the direct assay of LDL-captured PrP 27–30 and from the sandwich CDI of released PrP 27–30 were fitted to the Hill equation (see Eq. **1** in *Supporting Text*, which is published as supporting information on the PNAS web site) as a function of apoB concentration in LDL (Fig. 1). The results indicate that native human PrP 27–30 binds to LDL with positive cooperativity, with an apparent binding affinity (K_d^{app}) in the range of 30–60 pM (Fig. 1 and Table 1). Similar values were obtained with purified VLDL (data not shown). The Hill coefficient data indicate a potential stoichiometry of three to four HuPrP^{Sc} molecules binding to one LDL.

High-Affinity Capture of sCJD Prions by apoB. Because the principal protein component of both VLDL and LDL is apoB, we examined the ability of apoB to capture PrP 27–30 in sCJD brain homogenates or PTA precipitates. Although the binding of rPrP^{Sc} to apoB displayed positive cooperativity similar to that seen with the LDL and VLDL particles, the K_d^{app} values were reduced 4- to 10-fold (Fig. 3 and Table 1). These data suggest that apoB may be the binding partner for PrP^{Sc} in both VLDL and LDL, but that lipid components influence binding affinity and possibly stoichiometry.

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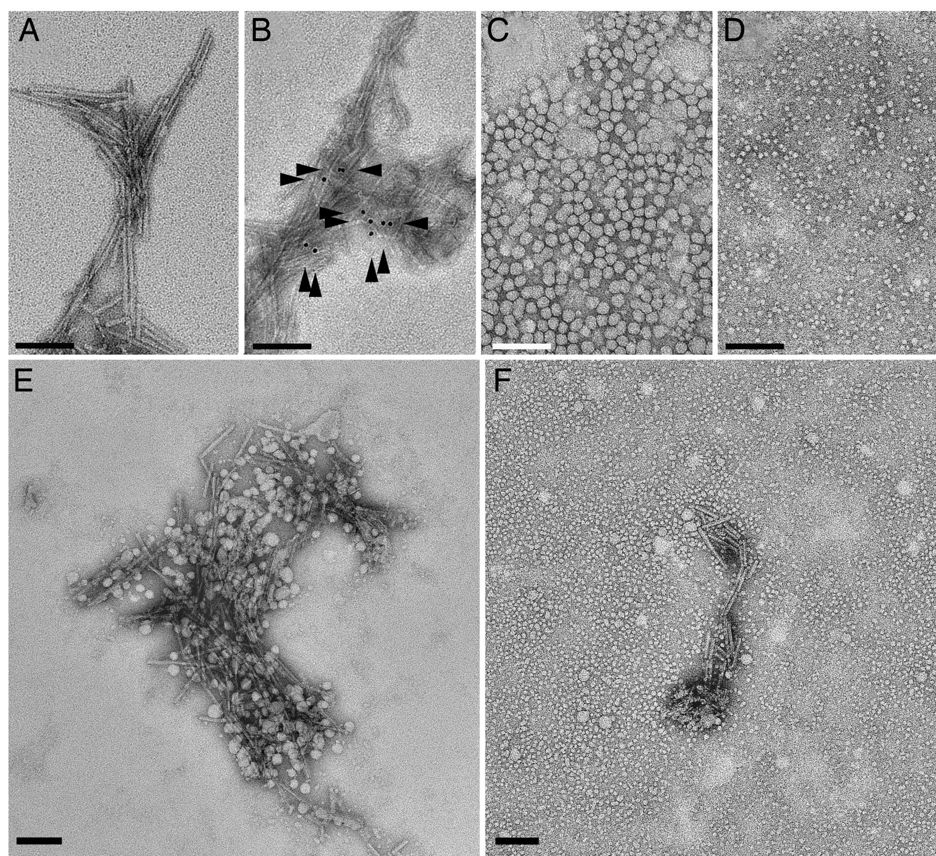

Fig. 2. Electron micrographs show decoration of prion rods purified from sCJD brain by human LDL. (*A*) Negative staining of PrP 27–30 polymerized into rods that were purified by PTA precipitation. (*B*) Prion rods decorated with α-PrP recHuM Fab R2 (66) and a 10-nm gold binary detection system (arrowheads). (*C*) Negatively stained LDL particles with an average size of 22 ± 2 nm. (*D*) Negatively stained HDL particles with an average size of 9 ± 1.5 nm. (*E*) Incubation of PrP 27–30 with LDL shows that almost all LDL particles are either in direct contact with or in close proximity to the prion rods. (*F*) Incubation of PrP 27–30 with HDL shows that virtually no HDL particles bind to the prion rods. HDL particles are randomly distributed over the grid surface. (Scale bars, 100 nm.)

Reduced Binding of α-Helical Human recPrP(90–231) to LDL and apoB. Because ≈50% of HuPrP 27–30 exposed to 4 M Gdn·HCl at 80°C for 20 min remained bound to LDL particles (Fig. 1*B*), we examined the binding of recombinant HuPrP composed of residues 90–231, designated recHuPrP(90–231). The binding of recHuPrP(90–231) to LDL was measured by using the CDI (Fig. 4): recHuPrP(90–231) was either folded into an α-helical conformation (10) or denatured with 4 M Gdn·HCl at 80°C for 20 min. Although α-helical recHuPrP(90–231) did not show appreciable binding to LDL, denatured recHuPrP did bind to LDL. In another set of experiments, four different concentrations of denatured recHuPrP(90–231) bound to apoB (Fig. 6, which is published as supporting information on the PNAS web site).

The series of isotherms for serially diluted recHuPrP(90–231) captured by LDL or apoB were fitted to the Hill equation as a function of concentration. Denatured recHuPrP(90–231) bound to both LDL and apoB with positive cooperativity and K_d^{app} values ranged from 70 to 90 pM (Table 1). The Hill coefficient data indicate a stoichiometry of ≈1 denatured recHuPrP(90–231) molecule binding to one LDL particle or

Table 1. Lipoproteins capture human PrP with high affinity (K_d^{app}) and positive cooperativity (σ)

HuPrP	Source	Conformation	Lipoprotein	σ*	K_d^{app}, pM
PrP 27–30	sCJD PTA	β-Sheet	ApoB	3.04 ± 0.00	208 ± 95
			Lip a	3.90 ± 3.72	46 ± 14
			LDL	3.89 ± 1.37	28 ± 3
	sCJD BH	β–Sheet	ApoB	2.14 ± 0.00	212 ± 59
			LDL	3.54 ± 0.00	58 ± 7
recHuPrP(90–231)	Recombinant	Random coil	ApoB	1.60 ± 0.05	94 ± 2
			Lip a	1.14 ± 1.35	33 ± 36
			LDL	1.34 ± 0.28	70 ± 5
	Recombinant	α-Helical	LDL	ND	>2,900

The raw fluorescence data were fit to the Hill function (Eq. **1** in *Supporting Text*), for which K_d^{app} is the apparent equilibrium affinity constant of PrP binding to the lipoprotein. The constant σ and K_d^{app} are an average ± SD calculated from three or four experiments at different concentrations of PrP (see Fig. 5).

*Hill coefficient describing the observed stoichiometry of positive cooperative binding.

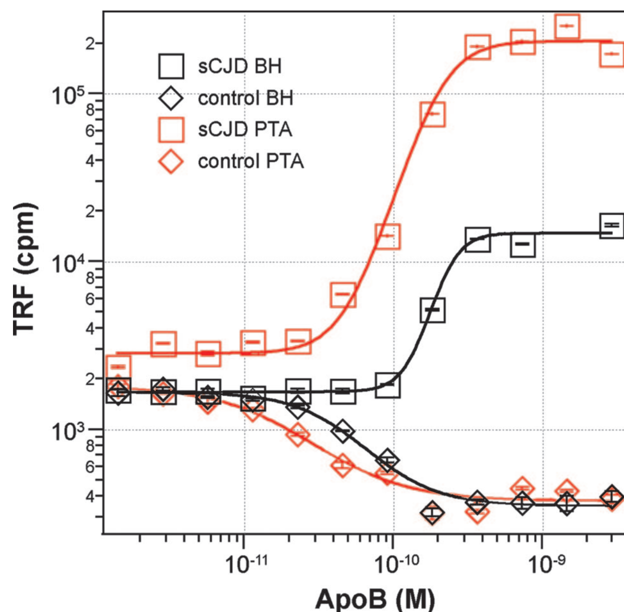

Fig. 3. Capture of sCJD prions by apoB. rPrP^{Sc} in brain homogenates (sCJD/BH) treated for 1 h at 37°C with 10 μg/ml PK or in fractions purified from sCJD brain homogenates by precipitation with 0.32% PTA and 2.6 mM $MgCl_2$ (sCJD/PTA) were diluted 20-fold and incubated on plates previously coated with serially diluted apoB. The captured rPrP^{Sc} was denatured on the plates and detected with Eu-labeled mAb 3F4 as described in the legend of Fig. 1 and *Materials and Methods*. The time-resolved fluorescence (TRF) values (average ± SD) are from three experiments and are directly proportional to the concentration of PrP 27–30.

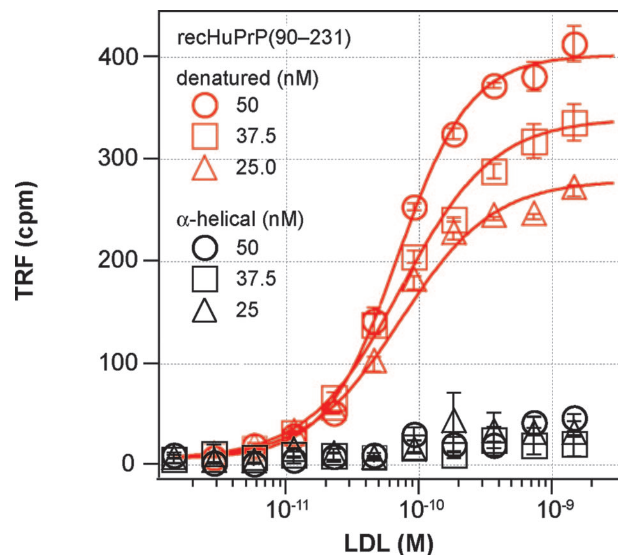

Fig. 4. Denatured recombinant human PrP binds to LDL. Denatured recHuPrP(90–231) but not α-helical recHuPrP(90–231) bound to LDL. Several concentrations of recHuPrP(90–231), either refolded into an α-helical conformation or denatured with 4 M Gdn·HCl at 80°C for 20 min, were incubated on plates coated with serial dilutions of LDL and then assayed by CDI. The time-resolved fluorescence (TRF) values (average ± SD) are from three experiments and are proportional to the concentration of PrP.

apoB protein. We conclude from these experiments that the PrP epitope for binding to apoB and LDL is within residues 90–231, and that the order of binding among the different PrP conformers tested is β-rich PrP 27–30 → denatured recHuPrP(90–231) → α-helical recHuPrP(90–231). The different stoichiometries observed for PrP 27–30 and denatured recHuPrP(90–231) binding to LDL suggest that β-rich PrP 27–30 has multiple sites, probably created by aggregation, that can interact with LDL.

Discussion

The lipophilic nature of PrP^{Sc} is similar to that of the apolipoproteins. Numerous attempts to remove the lipids bound to native PrP^{Sc} and substitute nondenaturing detergents have been unsuccessful (11, 12). Functional solubilization was achieved by dispersion of PrP^{Sc} into liposomes (13). Although the copartitioning of PrP^{Sc} and LDL was not surprising, the cooperative binding of PrP^{Sc} to LDL and apoB, with such high affinities (Table 1), but not to HDL, was unexpected (Figs. 1 and 2).

When PrP^{Sc} is formed from PrP^{C}, this process seems to occur in cholesterol-rich microdomains often referred to as rafts or caveolae-like domains (CLDs; see ref. 14). Rafts or CLDs enriched for PrP^{C} and PrP^{Sc} have been isolated by flotation gradients (15, 16). Depletion of cholesterol from cultured cells was found to inhibit the formation of nascent PrP^{Sc} (14).

Prions in Blood. Recently, variant CJD that is found in teenagers and young adults (17) appears to have been transmitted from prion-infected donors to three transfusion recipients (18–20). Such findings are consistent with earlier reports of low levels of prions in blood (21–23) and the replication of prions in the lymphoreticular system (24–29). The distribution of prions in blood has been difficult to resolve, because the levels of infectivity are so low (21, 30–32). Whether some prions are nonspecifically bound to circulating lymphocytes, perhaps by PrP^{C}, or whether they reproduce at low levels in a distinct set of lymphoid cells is unclear (33–36). Notably, transplantation of bone marrow from WT mice restored prion replication in the spleens of *Prnp*$^{0/0}$ mice (27).

In contrast to studies on white blood cells summarized above, our results suggest a measurable fraction of prion infectivity is likely to be associated with VLDL and LDL particles. Should prion infectivity be found in the VLDL or LDL fractions, then the utility of leukodepletion (32, 37) will need to be reconsidered.

Our finding that PrP^{Sc} binds to LDL particles is not the first report of PrP^{Sc} binding to a plasma protein. Other investigators reported mouse Rocky Mountain Laboratory (RML) prions bind to plasminogen (38), but subsequent studies by others demonstrated that the affinity of PrP for plasminogen is low (M. Vey, personal communication; see ref. 39). Additionally, studies in transgenic and knockout mice indicate that plasminogen expression does not influence the prion replication rate or the pathogenesis of RML prion infection (40).

Lipoproteins and Neurodegenerative Diseases. Although the function, if any, of lipoproteins in the pathogenesis of prion disease is unknown, the role of apolipoprotein E (apoE) in modulating the onset of Alzheimer's disease is well documented (41). Attempts to correlate the apoE isotype with sCJD were unsuccessful (42, 43), and apoE-deficient mice exhibited incubation times for RML prions that were indistinguishable from those in WT mice (44).

Receptors for both apoE and apoB have been identified in the CNS, but apoB is not expressed in brain (45). Each human LDL particle contains ≈3,000 lipid molecules and one apoB protein consisting of 4,536 amino acids (46). LDLs are composed of several distinct size and density subclasses (47); whether PrP^{Sc} preferentially binds to a particular LDL subclass remains to be determined.

Because >50 mutations in the apoB gene have been recorded, it will be of interest to determine whether any modify the age of onset, duration of the clinical course, or the constellation of

neurologic deficits in CJD. Many apoB mutants have been identified that diminish the binding of apoB to LDL receptors (48, 49).

The affinity of native PrP^{Sc} for apoB appears to be much greater than that of any PrP^{Sc}-specific antibody or aptamer reported to date (50–53). The binding of native PrP^{Sc} to apoB was enhanced up to 10-fold by the additional lipids found in LDLs (Table 1). This increase may be due to conformational changes in either apoB or PrP^{Sc} that are induced by the additional lipids (54).

It will be of interest to determine whether mixing naturally occurring or synthetic prions (55) with LDL before inoculation will alter the incubation time after intracerebral (i.c.) or i.p. inoculation. Whether LDL particles or a subclass will facilitate retention of prions in the brain after i.c. inoculation is unknown. In other investigations, we found that only ≈1% of injected prions can be found in the brain 48 h after i.c. inoculation (56). It will also be important to assess whether partitioning of prions into LDL particles alters the strain-specified properties of a particular inoculum.

Diagnostic Tests for Prions. Our findings are encouraging with respect to the development of a reliable antemortem test for prions. Perhaps the sensitivity and reliability of an antemortem blood test for prions can be increased by fractionation of LDLs into one or more of the subclasses noted above. How early in the disease course prions will be detected in sCJD cases is unknown; likewise, the time at which prions can be detected in LDL particles in familial cases of prion disease remains to be established. Whether such a blood test will be equally applicable to all mammals is also uncertain.

Whether all VLDL and LDL particles should be removed from human blood and plasma that is used in transfusions remains to be determined. Such an approach may find application in some higher-risk populations, such as in Britain, where it has been estimated that thousands of people are replicating variant CJD prions in their lymphoid tissues (28).

It should be possible to engineer a mutant apoB protein–lipid complex with a very high affinity for PrP^{Sc}. Such a mutant apoB might find application first as a capture moiety in a prion diagnostic test like the CDI and then as a pharmacotherapeutic. Whether an i.v.-administered apoB mutant might function analogously to anti-Aβ antibodies being used in the treatment of Alzheimer's disease remains to be established (57–59). Interestingly, expression of human apoE in transgenic mice reduced Aβ deposition in a model of Alzheimer's disease (60).

Materials and Methods

The LDL, HDL, and VLDL fractions from healthy donors were purified as described in *Supporting Text* (61).

The preparation and purification of recHuPrP(90–231) were as described for SHaPrP(90–231) (see ref. 10). The molecular mass for recHuPrP(90–231) was 16,059 Da, as determined by MS. The refolding of recHuPrP(90–231) into an α-helical protein is described in *Supporting Text*.

The CDI data described in this paper were generated with Eu-labeled mAb 3F4 (62, 63) or recHuMFab P (64).

Partitioning of CJD prions during fractionation of human plasma spiked with sCJD brain homogenate was analyzed by using a modified protocol for purification of lipoproteins (6). The details of the plasma fractionation using PTA and $MgCl_2$ are described in *Supporting Text*.

Electron microscopy (65) used formvar/carbon-coated 200-mesh copper grids that were glow-discharged before staining. The grids were negatively stained with ammonium molybdate. Immunolabeling was performed by using the recHuMFab R2. Details are described in *Supporting Text*.

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