Aggregated prion protein (PrP^Sc), which is detergent-insoluble and partially proteinase K (PK)-resistant, constitutes the major component of infectious prions that cause a group of transmissible spongiform encephalopathies in animals and humans. PrP^Sc derives from a detergent-soluble and PK-sensitive cellular prion protein (PrP^C) through an α-helical to β-sheet transition. This transition confers on the PrP^Sc molecule unique physicochemical and biological properties, including insolubility in non-denaturing detergents, an enhanced tendency to form aggregates, resistance to PK digestion, and infectivity, which together are regarded as the basis for distinguishing PrP^Sc from PrP^C. Here we demonstrate, using sedimentation and size exclusion chromatography, that small amounts of detergent-insoluble PrP aggregates are present in uninfected human brains. Moreover, PK-resistant PrP core fragments are detectable following PK treatment. This is the first study that provides experimental evidence supporting the hypothesis that there might be silent prions lying dormant in normal human brains.

Human prion diseases may be sporadic, familial, or acquired by infection, and include four major phenotypes: Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker disease, fatal familial insomnia, and kuru (1). The central event in the pathogenesis of all forms of prion disease involves a conversion of the host-encoded cellular prion protein PrP^C to its pathogenic conformer PrP^Sc (2). Whereas PrP^C is detergent-soluble and sensitive to proteinase K (PK) digestion, PrP^Sc forms detergent-insoluble aggregates and is partially resistant to PK (3–7). The conversion of PrP^C to PrP^Sc is known to involve a conformational transition of an α-helical to a β-sheet structure of the protein (8–10) but the in vivo pathway is still poorly understood. Two non-exclusive conversion models have been put forward: refolding (11, 12) and seeding (13). In the refolding model, the exogenous PrP^Sc binds to a PrP^C species that has been partially unfolded and the PrP^Sc-bound PrP^C molecule undergoes a refolding process, during which nascent PrP^Sc is derived from this PrP^C species via a conformational transition. The seeding or nucleation-polymerization model proposes that a small amount of abnormal PrP^Sc or PrP^Sc precursor (PrP*) is present in the normal brain and is in reversible equilibrium with PrP^C. When several precursor monomeric PrP^* molecules form a highly ordered nucleus, PrP^* can be converted to PrP^Sc polymers. Clearly, two key elements are required by the seeding model. One is the presence of a small amount of endogenous PrP^Sc or PrP* in the uninfected brain and the second is the formation of PrP^Sc-derived oligomers. The seeding model, with the two elements, has been recapitulated in vitro using PrP from various fungal and mammalian sources (14–18). However, this hypothetical endogenous PrP^Sc, PrP* or “silent prion” has yet to be identified in the uninfected brains (13, 19, 20).

We isolated and characterized insoluble PrP aggregates (designated iPrP) from uninfected human brains using sedimentation and size exclusion chromatography. Our data demonstrate that this isoform accounts for about 5–25% of total PrP including full-length and N-terminal truncated forms present in uninfected brains and that it can be captured by Fd gene 5 protein (g5p) and sodium phosphotungstate (NaPTA), two reagents that specifically bind to PrP^Sc but not to PrP^C (21, 22). The state of the various PrP species range from a monomer to small oligomers (less than 200 kDa), to large aggregates (equal to or greater than 2,000 kDa). Moreover, we observed in these brains protease-resistant PrP core fragments migrating at ~20, 18–19, and ~7 kDa on immunoblots, after PK digestion and deglycosylation using anti-PrP antibodies. These PK-resistant PrP fragments are derived from the detergent-insoluble fraction (P2) and are present in the fraction captured with g5p.

## EXPERIMENTAL PROCEDURES

Reagents and Antibodies—NaPTA, PK, and phenylmethylsulfonyl fluoride were purchased from Sigma. Peptide N-glycosidase F (PNGase F) was purchased from New England Biolabs...
(Beverly, MA) and used following the manufacturer’s protocol. Urea, CHAPS, dL-dithiothreitol, iodoacetamide, tributylphosphine, Ampholine pH 3–10, and immobilized pH gradient (IPG) strips (pH 3–10, 11 cm long), and antibody stripping solution were from Bio-Rad (Richmond, CA). Reagents for enhanced chemiluminescence (ECL Plus) were from Amer sham Biosciences, Inc. Magnetic beads (Dynabeads M-280, tosyl-activated) were from Dynal Co. (Oslo, Norway). Anti-PrP antibodies, including rabbit antisemur (anti-C) immunoreactive to human PrP residues 220–231 (23), mouse monoclonal antibody 3F4 against human PrP residues 109–112 (24), mouse monoclonal antibody 1E4 against human PrP-(97–108) (Cell Sciences, Inc., Canton, MA), and 6H4 against human PrP-(144–152) (Prionics AG, Switzerland) were used. Rabbit antica veeolin-1 polyclonal antibody was from Clontech.

Preparation of Gene 5 Protein (g5p)—The g5p was isolated from Escherichia coli TG1(f−) cells following infection with fd bacteriophage. Purification was performed using DNA cellulose affinity and CM-ion exchange chromatography as described (25). The purity was >99% as determined by quantitation of Coomassie Blue-stained bands on SDS-PAGE.

Brain Tissues—Consent to use autopsy material for research purposes was obtained for all human brain samples. Autopsy was performed within 20 h from death. Biopsy brain tissues were frozen immediately in liquid nitrogen and then transferred to −80 °C for future use. Clinical data and relevant hospital records were examined. The normal human brains were obtained from subjects free of neurological disorders and PrP mutations as indicated by neurohistology, immunohistochemistry, Western blotting, and genetic analysis at the National Prion Disease Pathology Surveillance Center (Cleveland, OH). Six cases including 4 autopsies and 2 biopsies were used and the average age at death was 61 ± 10 years (49–79 years). In addition, 14 scJD cases were used as controls. The uninfected brain tissues from transgenic mice expressing 2-fold human PrP (26), hamster, and cow were also used in this study.

Preparation of Brain Homogenate and Detergent-soluble (S2) and -Insoluble (P2) Fractions—The 10% (w/v) brain homogenates were prepared in 9 volumes of lysis buffer (10 mM Tris, 150 mM NaCl, 0.5% Nonidet P-40, 0.5% deoxycholate, 5 mM EDTA, pH 7.4) on ice using pestles with microtubes driven by a cordless motor. When required, brain homogenates were centrifuged at 1,000 × g for 10 min at 4 °C to collect supernatant (S1). To prepare S2 and P2 fractions, S1 were further centrifuged at 35,000 rpm (100,000 × g) in an SW55 rotor (Beckman Coul ter, Fullerton, CA) for 1 h at 4 °C. After ultracentrifugation, the supernatants that contain the detergent-soluble fraction were transferred into a clean tube. After being washed gently with 1× lysis buffer twice to remove residual supernatant proteins, the pellets that contain detergent-insoluble fraction (P2) were further resuspended in the lysis buffer as described (27).

Velocity Sedimentation in Sucrose Step Gradients—Supernatant prepared by centrifugation of 20% brain homogenate at 1,000 × g for 10 min at 4 °C was incubated with an equal volume of 2% Sarcosyl for 30 min on ice. The sample was loaded atop 10–60% step sucrose gradients and centrifuged at 200,000 × g in the SW55 rotor for 1 h at 4 °C as described with minor modifications (28, 29). After centrifugation, the contents of the centrifuge tubes were sequentially removed from the top to the bottom to collect 12 fractions. Aliquots of 12 fractions were subjected to immunoblot analysis as described below.

Size Exclusion Chromatography—Superdex 200 HR beads (GE Healthcare) in a 1 × 30-cm column were used to determine the oligomeric state of PrP molecules. Chromatography was performed in an fast protein liquid chromatography system (GE Healthcare) at a flow rate of 0.25 ml/min and fractions of 0.25 ml each were collected as described (28). In brief, 200-µl samples, prepared as described above (sucrose step gradients), were injected into the column for each size exclusion run. The molecular weight (Mw) of the various PrP species recovered in different fast protein liquid chromatography fractions was evaluated according to a calibration curve generated with the gel filtration of molecular mass markers (Sigma) including dextran blue (2,000 kDa), thyroglobulin (669 kDa), apoferritin (443 kDa), β-amy lase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), and carbonic anhydrase (29 kDa). These standards were loaded independently at the concentrations recommended by Sigma in 200-µl sample volumes. The elution volume of dextran blue was used to determine the void volume (V0 = 8.45 ml) and the total volume (Vt = 24 ml) was provided by the product instruction. The peak elution volumes (Vp) were calculated from the chromatogram and fractional retentions. Ksv were calculated using the equations: Ksv = (Vp − V0)/(Vt − V0) (30). The calibration curve was determined by plotting the Ksv of the protein standards against the log Mw of the standards (30).

Capture of PrP by g5p—The g5p molecule (100 µg) was conjugated to 7 × 108 tosyl-activated magnetic beads in 1 ml of phosphate-buffered saline (PBS) at 37 °C for 20 h (21). The g5p-conjugated beads were incubated with 0.1% bovine serum albumin in PBS to block nonspecific binding. The prepared g5p beads were stable for at least 3 months at 4 °C. The capture of PrP by g5p was performed as described (21) incubating S1 fractions or P2 with g5p-conjugated beads (10 µg of protein/6 × 107 beads) in 1 ml of binding buffer (3% Tween 20, 3% Nonidet P-40 in PBS, pH 7.5). After incubation with constant rotation for 3 h at room temperature, the PrP-containing g5p beads were attracted to the sidewall of Eppendorf tubes by external magnetic force, allowing easy removal of all unbound molecules in the solution. Following three washes in wash buffer (2% Tween 20 and 2% Nonidet P-40 in PBS, pH 7.5), the g5p beads were collected and heated at 95 °C for 5 min in SDS sample buffer (3% SDS, 2 mM EDTA, 10% glycerol, 50 mM Tris-HCl, pH 6.8).

Precipitation of PrP by NaPTA—Precipitation of PrP by NaPTA was conducted as described (31) with minor modifications. Briefly, 10% (w/v) homogenates from brain tissues were prepared in PBS lacking Ca2+ and Mg2+. The samples were centrifuged at 1,000 × g for 10 min at 4 °C. A 500-µl aliquot of supernatant was mixed with an equal volume of 4% (w/v) Sarcosyl prepared in PBS, pH 7.4, and incubated for 10 min at 37 °C with constant agitation. Samples were adjusted to final concentrations of 50 units/ml Benzonase (Benzonuclease, Merck & Co, Whitehouse Station, NJ) and 1 mM MgCl2 and incubated for 30 min at 37 °C with constant agitation. Subsequently, the samples were adjusted to a final concentration in the sample of
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0.3% (w/v) NaPTA with 81.3 μl of a stock solution containing 4% (w/v) NaPTA and 170 mM MgCl₂. Samples were incubated at 37 °C for 30 min with constant agitation before centrifugation at 16,000 × g for 30 min. After removal of the supernatant, the pellet was resuspended in 1× lysis buffer for Western blotting as described below.

One- and Two-dimensional Gel Electrophoresis and Immunoblotting—Samples were resolved either on 15% Tris-HCl Criterion pre-cast gels (Bio-Rad) for one-dimensional gel electrophoresis or IPG strips for the two-dimensional gel electrophoresis.

Two-dimensional gel electrophoresis was performed as described by the supplier using the PROTEIN IEF cell (Bio-Rad) (27, 32). Samples denatured by boiling in SDS sample buffer were incubated with reducing buffer (8 M urea, 2% CHAPS, 5 mM tributylphosphine, 20 mM Tris, pH 8.0) for 1 h at room temperature and then incubated with 200 mM iodoacetamide for 1 h. Proteins were precipitated with a 5-fold volume of pre-chilled methanol at −20 °C for 2 h and centrifuged at 16,000 × g for 20 min at 4 °C. The pellets were resuspended in 200 μl of rehydration buffer (7 M urea, 2 M thiourea, 1% dithiothreitol, 1% CHAPS, 1% Triton X-100, 1% Amphotile pH 3–10, and trace amounts of bromophenol blue). The pellets were dissolved in rehydration buffer and subsequently incubated with the IPG strips for 14 h at room temperature with gentle shaking.

The dehydrated gel strips were transferred onto a focusing tray and focused for about 40 kV·h. The focused IPG strips were equilibrated for 15 min in equilibration buffer 1 (6 M urea, 2% SDS, 20% glycerol, 130 mM dithiothreitol, 375 mM Tris-HCl, pH 8.8), and then another 15 min in equilibration buffer 2 (6 M urea, 2% SDS, 20% glycerol, 135 mM iodoacetamide, 375 mM Tris-HCl, pH 8.8). The equilibrated strips were loaded onto the 8–16% Tris-HCl Criterion pre-cast gel (Bio-Rad).

The proteins on the gels were transferred to Immobilon-P membrane polyvinylidene fluoride (Millipore) for 2 h at 70 V.

For probing of the PrP or caveolin-1, the membranes were incubated for 2 h at room temperature with anti-PrP antibodies including 3F4 (1:40,000), 1E4 (1:500), anti-C (1:4,000), 6H4 (1:10,000), or anti-caveolin-1 (1:5,000) as the primary antibody. Following incubation with horseradish peroxidase-conjugated sheep anti-mouse IgG or donkey anti-rabbit IgG at 1:3,000, the PrP or caveolin-1 bands or spots were visualized on Kodak film by ECL Plus as described by the manufacturer.

RESULTS

Detergent-insoluble PrP Species Are Present in Uninfected Human Brains—It has been shown that PrPc is recovered in a soluble fraction (S2), following ultracentrifugation in non-denaturing detergents at 100,000 × g for 1 h at 4 °C, whereas PrPSc is recovered in an insoluble fraction (P2) (6, 7, 23). When we applied this separation procedure to human brain tissues, free of prion diseases (non-PrD), as expected, most of the PrPc was recovered in the S2 fraction (Fig. 1, A and B). Surprisingly, a small amount of PrP was consistently detectable in the P2 fraction (Fig. 1, A and B). Quantitative densitometric analysis of immunoblots probed with 3F4 antibody showed that 96% of the PrP detected was recovered in S2 as a soluble form, whereas 4% of the PrP was recovered in P2 as a detergent-insoluble form (mean ± S.D.: 96 ± 2 versus 4 ± 2%, n = 6) (Fig. 1A). An increased intensity of PrP staining was observed in P2 on the blot re-probed with anti-C (mean ± S.D.: 75 ± 9 (S2) versus 25 ± 9% (P2), n = 6) (Fig. 1B), suggesting that predominant PrP distributed in P2 is N-terminal truncated. Taken together, PrP recovered in P2 accounted for ~5–25% of total PrP including full-length and N-terminal-truncated species.

This novel isofrom, termed insoluble PrP (iPrP), had a banding profile different from that of soluble PrPc in the immunoblot probed with 3F4 (Fig. 1, A and C). In normal human brains, PrPC from S2 always possesses a dominant upper band. The intensity of the middle PrP band is often similar or less than that of the upper band. However, the intensity of its low band normally is the lowest among the three PrP bands (Fig. 1C, middle panel). By contrast, the ratio of the low to upper PrP band of the iPPrP species significantly increased, which is similar to that found with PrPSc (1) (Fig. 1C, left and right panels). Following PK treatment, the PK-resistant core fragment (termed PrPc (27–30)) was only detected with 3F4 in PrPSc, but not in either iPPrP or PrPc (Fig. 1C).

Uninfected Human Brains Contain PrP Aggregates That Are Typically Detected in Prion-infected Brains—It is conceivable that the iPPrP molecules also form aggregates in uninfected human brains. To investigate this possibility, velocity sedimentation of PrP in sucrose step gradients from 10 to 60% was conducted. Various PrP species including PK-sensitive aggregates of heterogeneous sizes can be separated by this procedure...
into several fractions based upon distinct molecular densities, sizes, and shapes (28, 29). The abundant PrP from the brains of non-PrD was distributed through the upper fractions from 1 to 4 (Fig. 2A). By contrast, PrP from brains infected with prion disease was predominantly recovered in the bottom fractions from 9 to 12 (Fig. 2B). The distribution profile of PrP from non-PrD and sCJD was significantly different. The majority of PrP species recovered in the top fractions from non-PrD represent PrPC that consisted mainly of detergent-soluble monomers. The increased PrP in fractions 9–12 from sCJD indicate that PrPSc formed large aggregates that fractionated toward the bottom of the sucrose gradients (28, 29).

However, small amounts of PrP were also consistently detectable in the bottom fractions derived from the brains of non-PrD. The amount of PrP aggregates precipitating in bottom fractions 9–12 (Fig. 2, A and C) accounted for ~5% of the total PrP in non-PrD (n = 6), similar to the amount of iPrP detected with 3F4 in fraction P2 (Fig. 1). By contrast, the PrPSc aggregates in fractions 9–12 from sCJD brain homogenates (n = 4) accounted for ~50.4% of the total PrP (Fig. 2, B and C). The high reproducibility of finding of PrP aggregates in 6 non-PrD brains including 2 biopsy brain samples makes it unlikely that they are a product of postmortem autolysis.

We then conducted a comparison of PrP levels in the top and bottom fractions between non-PrD and sCJD on a single blot, which should determine more precisely the changes in the exact amounts of the PrP species in these fractions. Equal volumes of samples from either the top (fraction 1), or the bottom (fraction 9), of a non-PrD and two sCJD cases, were each loaded into the same gel. Compared with non-PrD, the level and/or banding pattern of PrP in fraction 1 (top) for sCJD was profoundly different, in addition to a significant increase in the amount of PrP in fraction 9 (bottom) (Fig. 2D). Therefore, in the brains with non-PrD, whereas most PrP species consisting of monomeric PrPC were recovered in the top fractions of sucrose gradients, there were also small amounts of PrP recovered in the bottom fractions. Because these PrP species were present in all bottom fractions, the density and size must have increased continuously, which is an indication of aggregation of the PrP (28, 29). By contrast, in the sCJD-affected brains, the PrP species in the bottom fractions significantly increased, whereas PrP in the top fractions decreased, indicating that most of the PrP species was in aggregate form, along with a decrease in the level of PrPC, a result consistent with our recent study on characterization of PrP in the most common subtype of sCJD (33).

In addition, we also investigated hamsters and cows to see if the finding of the presence of iPrP in uninfected human brains is seen in other animals. A small amount of PrP was also detect-
able in the sucrose gradient bottom fractions of uninfected cow and hamster brains (Fig. 2, E and F).

We next examined another membrane protein caveolin-1, as a control, to confirm that this assay is able to faithfully determine the oligomeric state of a membrane protein. Caveolin-1 is mainly localized in plasma-membrane caveolae and may also have soluble cytoplasmic and secreted forms (34). It has been demonstrated that both recombinant and endogenous caveolin-1 form high molecular mass oligomers of 200–400 kDa in vitro and in vivo (35, 36). After stripping 3F4 from used Western blots, we re-probed the blots with rabbit anti-caveolin-1 polyclonal antibody. Caveolin-1 from both uninfected and prion-infected human brains was mostly distributed in fractions 2–6, although it was also observed in other fractions (Fig. 3, A and B). Compared with non-PrD, sCJD had higher levels of caveolin-1 in fractions 2–6. Therefore, in the uninfected human brains the membrane protein caveolin-1 is mainly present as small oligomers and small amounts of large aggregates are also detectable, which is different from the membrane protein PrPC. Prion-infected human brain showed an increase in the amounts of only oligomeric caveolin-1 but not large caveolin-1 aggregates. This is inconsistent with the distribution of PrP.

The Size of iPrP Aggregates in Uninfected Human Brains Is Similar to That of PrPSc Aggregates Present in Prion-infected Brains—The size of the various PrP conformers of non-PrD samples was further characterized using size exclusion chromatography (also called gel filtration). We first generated a calibration curve with seven molecular mass markers from Sigma (Fig. 4A). We then examined the $M_r$ of PrP from non-PrD and sCJD. PrP from sCJD was mainly eluted in two groups of fractions with distinct retention times: the first group appeared before fraction 33 and the second after fraction 49 (Fig. 4B). Based on the sizing calibration curve (Fig. 4A), fractions 27–33 contained PrP aggregates equal to or greater than ~2,000 kDa and fractions 49–61 contained monomers and small oligomers less than 200 kDa (Fig. 4B). Although most PrP from non-PrD was eluted after fraction 49, small amounts of PrP were detectable in fractions 27–47 (Fig. 4C), indicating that uninfected brains also contain small quantities of various types of PrP aggregates. Compared with non-PrD, all 14 sCJD cases examined by gel filtration showed an increase in the PrP level before fraction 33 (Fig. 4B), suggesting that all sCJD have an increased PrP aggregation. However, PrP levels in the two groups (after fraction 49 and before fraction 33) were similar in seven of the 14 sCJD cases (Fig. 4B). It was noted that sCJD had more PrP species with a high $M_r$ in the sucrose gradient bottom fractions, compared with the early fractions, based on size exclusion chromatography. This could be because sucrose gradients recover all PrP species tested, whereas size exclusion chromatography has low efficiency for the recovery of larger aggregates of molecular mass greater than 2,000 kDa, due to the limited column capability.

Small Amounts of PrP Are Captured or Precipitated from Uninfected Human Brains by Specific PrPSc-binding Reagents—We have recently demonstrated that g5p, a single-strand DNA-binding protein, is capable of specifically capturing PrPSc from prion-infected human and animal brains (21). The binding of g5p was supposed to occur either to a PrPSc-DNA complex or to PrPSc aggregates, which may have a structure similar to DNA (21). We then determined whether any PrP conformers could be captured by g5p from a relatively large amount of non-PrD brain homogenates, and whether captured PrP is PK resistant.
By contrast to the case of PrPSc from sCJD, a trace of PrP from a relatively large volume of samples was also captured in 6 non-PrD (sCJD versus non-PrD: 1 versus 100 μl) (Fig. 5A). Although a 100-fold larger volume of sample from non-PrD was used for each case, the amount of PrP captured was much less than that of PrPSc captured from a sCJD control (Fig. 5A). PrP capture was probably not due to nonspecific binding, because in the immunoblot, the banding pattern of g5p-captured PrP from non-PrD was different from that of PrPSc (Figs. 1C and 5A). Compared with PrPSc, the ratio of the intensity of the low to the upper PrP band was significantly increased. Furthermore, when PrP was denatured in the SDS sample buffer, prior to g5p capture, no detectable PrP was captured with g5p from the denatured sample (Fig. 5A, lane 8).

NaPTA is widely used to precipitate detergent-insoluble PrP aggregates (22, 31). Using NaPTA, even more PrP was precipitated from 6 non-PrD cases compared with the amount of PrP captured by g5p (sCJD versus non-PrD: 5 versus 250 μl) (Fig. 5B). This is consistent with the observation reported by Wadsworth et al. (31), in which less than 5% of total PrP was observed to be precipitated by NaPTA from uninfected human brains.

Whether the PrP species precipitated by g5p or NaPTA are equivalent to iPPr remains to be further determined. Nevertheless, these results provide additional evidence that uninfected human brains indeed contain small amounts of PrP species that can be captured by g5p or NaPTA, suggesting that these unique PrP conformers are different from the normal soluble PrPSc species. These unique conformers may have an abnormal structure with a high tendency to form aggregates.

The iPPr Molecule Has a Two-dimensional Gel Profile Similar to That of PrPSc—Two-dimensional gel electrophoresis is capable of separating proteins based on both molecular weight and charge, and has furthered our understanding of the composition of the three PrP bands on one-dimensional blots (37, 32). The middle band on the one-dimensional blot was previously considered to be full-length monoglycosylated PrP, however, using two-dimensional gel electrophoresis it has been demonstrated that it actually consists of a large amount of truncated diglycosylated PrP fragments and only a little full-length monoglycosylated PrP. Similarly, the low M, band seen on one-dimensional blots was considered to be unglycosylated full-length PrP, whereas using two-dimensional gel electrophoresis it has been found that this band also contains truncated monoglycosylated PrP fragments (37, 32).

As described above, the pattern of iPPr on a one-dimensional blot is similar to that of PrPSc, showing an increase in the relative intensity of middle and low PrP bands. We next further investigated the differences in the composition of PrP conformers among PrPSc, iPPr, and PrPSc using two-dimensional gel electrophoresis. The two-dimensional blot of PrPSc from P2 of sCJD is mainly composed of six sets of PrP spots, as we have recently shown (32). PrP spots I, II, and III correspond to full-length di-, mono-, and un-glycosylated PrP species, respectively, whereas PrP spots IV, V, and VI correspond to N-terminal truncated di-, mono-, and un-glycosylated PrP, respectively (Fig. 6C). By contrast, PrPSc recovered in the S2 fraction consisted mainly of three sets of PrP spots: spots I, II, and IV (Fig. 6A). Spot III of PrPSc was barely detectable. Although iPPr almost contained all spots except II, the ratio of these sets of PrP spots was different from that of PrPSc (Fig. 6B). PrP spot IV from iPPr was much higher than that from PrPSc, whereas all other PrP spots were lower from iPPr than from PrPSc. Compared with PrPSc, iPPr had increased levels of spots III, IV, V, and VI, and decreased levels of I and II. Following deglycosylation with PNGase F, two sets of deglycosylated PrP spots (III and VI) increased in all three conditions, representing full-length and N-terminal-truncated PrP species, whereas PrP spots I, II, IV, and V, representing glycosylated full-length and truncated PrP, became undetectable (Fig. 6, D–F). However, an additional set of PrP spots (III’ ) was present in the S2 sample from non-PrD, which could be a larger N-terminal-truncated PrP species (Fig. 6D). Compared with soluble PrPSc (Fig. 6D), insoluble PrP, from both non-PrD and sCJD, formed spots that had a much lower resolution (Fig. 6, E and F), suggesting that they were mainly composed of PrP aggregates. Therefore, iPPr possesses a two-dimensional profile that is similar to PrPSc. Fig. 6 is a representative of 3 non-PrD and 3 sCJD cases.

There Are PK-resistant Core Fragments in the Uninfected Human Brains—The results from the detergent-insolubility assay, the sedimentation in sucrose gradients, gel filtration, and g5p/NaPTA capture taken together indicate that small amounts of PrP aggregates were present in uninfected brains, suggesting that at least some of these PrP aggregates are PK-resistant. To test for this possibility, three anti-PrP antibodies
against different domains of human PrP were utilized. These were the widely used monoclonal antibody 3F4, against the human PrP amino acid sequence MKHM-(109–112) (24), a monoclonal antibody 1E4, against the human PrP amino acid sequence 97SQWNKPSKPKTN108 (Cell Sciences, Inc., Canton, MA), and a polyclonal antibody anti-C, against the human PrP amino acid sequence 220RESQAYYQRGSS231 (23).

In the untreated samples from both non-PrD and sCJD, all three antibodies recognized the three upper, middle, and low PrP bands (Fig. 7, A–C). In the PK-treated samples, 3F4 recognized the three partially PK-resistant core fragments of PrP-(27–30) only in sCJD, but not in the non-PrD sample, as expected (Fig. 7A). In the samples treated with PNGase F, 3F4 recognized two PrP bands from both non-PrD and sCJD: an upper band migrating at ~27–28 kDa, representing deglycosylated full-length PrP and a lower band migrating at ~20–21 kDa, representing deglycosylated N-terminal-truncated PrP fragment (Fig. 7A). In the samples treated with both PK and PNGase F, again 3F4 detected the deglycosylated PrP-(27–30) migrating at ~20–21 kDa only in the sCJD sample but not in non-PrD (Fig. 7A).

However, when the blot was re-probed with either 1E4 or anti-C antibody, two novel types of PK-resistant PrP core fragments were detected in the samples from non-PrD (Fig. 7, B and C). In the blot re-probed with 1E4, two faint PrP bands migrating at ~29–30 and ~19–20 kDa, respectively, were detectable in the non-PrD sample treated with PK alone (Fig. 7B). Following treatment with PK plus PNGase F, a single band migrating at ~20 kDa, designated PrP*20, to distinguish it from PrP-(27–30), was detectable. The intensity of this band was slightly increased compared with the sample without PNGase F treatment, suggesting that the upper band migrating at ~29–30 kDa could be a fully glycosylated form.
of PrP<sup>20</sup>. Using the anti-C antibody, the non-PrD sample treated with PK alone showed three PrP bands migrating between ~22 and ~30 kDa (Fig. 7C). Two bands remained after deglycosylation with PNGase F, one faint band migrating at ~20 kDa, corresponding to PrP<sup>20</sup> and another intense band migrating at ~18 kDa. The latter was designated PrP<sup>18</sup>. There was no detectable PrP band migrating at ~18 kDa before deglycosylation. Therefore, PrP<sup>18</sup> could be mostly glycosylated. PrP<sup>Sc</sup> from sCJD generated PrP-(27–30) and PrP-CTF12/13 upon treatment with PK and PNGase F, as expected (Fig. 7C). The PrP<sup>20</sup> fragment detected by both 1E4 and anti-C should include the 3F4 epitope, because the epitope of 1E4 (PrP-(97–108)) is localized next to the N terminus of the epitope of 3F4 (PrP-(109–112)). Moreover, compared with the intensity of PrP-(27–30) detected by the three antibodies, the affinity of 3F4 to the PK-resistant fragment was even slightly higher than that of 1E4 and anti-C. The inability of 3F4 to recognize PrP<sup>20</sup> suggests that PrP<sup>20</sup> may represent a unique PrP species possessing an unusual structure. Results shown in Fig. 7, A–C, were representative of a total of at least 6 non-PrD subjects.

To determine whether the two new PK-resistant PrP fragments were derived from S2 that mainly contained soluble PrP<sup>C</sup> or P2 that mainly contained the iPrP molecule, the S2 and P2 fractions were treated with PK and PNGase F, prior to SDS-PAGE and immunoblotting with 1E4 and anti-C. We observed that PrP<sup>20</sup> was detectable using 1E4 only in the P2 fractions from the four non-PrD cases and a transgenic mouse expressing PrP<sup>C</sup>. The fragment of 7–8 kDa (data not shown) became detectable in the P2 fractions of all 5 subjects, in addition to PrP<sup>20</sup> (Fig. 7E). This clearly demonstrated that the two PK-resistant PrP fragments were present in the detergent-insoluble P2 fraction in which the iPrP aggregates were identified.

Next we asked whether the PrP species captured by g5p is PK-resistant. To increase the yields of captured PrP, we used 50 μl of the P2 fraction, equivalent to 500 μl of S1 sample. Again, PrP<sup>20</sup> was observed in all three non-PrD cases. Moreover, two more PK-resistant PrP fragments, migrating at ~18–19 and ~7–8 kDa, were also detected in these non-PrD with 1E4 (Fig. 7F). Similarly, anti-C also detected PrP<sup>18</sup> and PrP<sup>20</sup> but not the PK-resistant fragment migrating at ~7–8 kDa (data not shown). The fragment of 7–8 kDa could be truncated at both the C and N termini of the protein.

Finally, we investigated the distribution of PK-resistant PrP species in sucrose step gradients. The PK-resistant core fragment PrP-(27–30), detected by 3F4, was present in all fractions from 1 to 12 but it was predominantly distributed in fractions 9–12, as shown in Fig. 8A. This result is in line with a previous observation in scrapie-infected mice (29). No detectable PrP-(27–30) was observed with 3F4 in sucrose gradient fractions from uninfected brains except for fraction 1, which contained small amounts of undigested PrP migrating at ~30–37 kDa (Fig. 8B). By contrast, when the blot was re-probed with 1E4 antibody following stripping, a PK-resistant PrP fragment migrating at ~20 kDa was observed in fractions 9–12, which corresponded to PrP<sup>20</sup>. Three PrP bands migrating at ~27–30, ~26–27, and ~20–21 kDa were observed in fraction 1, of which the upper band corresponding to the N-terminal cleaved diglycosylated PrP fragment was dominant (Fig. 8C). Therefore, the PrP aggregates at the higher density were composed of mainly unglycosylated PrP species, whereas the monomers or small oligomers of PrP, present in the top fractions, were comprised of diglycosylated PrP species.

**DISCUSSION**

Using a combination of biophysical and biochemical approaches, we have investigated solubility, size, density, and PK resistance of PrP from uninfected human brains. Our findings indicate that: 1) there are small amounts of insoluble PrP aggregates and PK-resistant PrP species in uninfected human brains and 2) in terms of solubility, aggregate size, density, and truncation of PrP in the untreated samples, the difference between prion-infected and uninfected brains seems to be quantitative rather than qualitative. These findings raise several issues and implications as to the structural and physicochemical properties of brain PrP<sup>C</sup>, the origin of PrP<sup>Sc</sup>, and the role of insoluble PrP aggregates in the pathogenesis of prion diseases.

Mature human PrP<sup>C</sup> is a glycoprotein encompassing residues 23–231. It contains a disulfide bond between two cysteine residues at positions 179 and 214 and is attached to the cell surface via a glycosylphosphatidylinositol anchor (2). NMR studies of recombinant human PrP-(23–230) have shown that the protein contains a flexible N-terminal domain from residues 23 to 124 and a folded C-terminal domain from residues 125 to 228, comprising two β-strands and three α-helices (38). Helix 2 (α2) and helix 3 (α3) in the C-terminal domain are linked by the disulfide bond. A crystal structure of the recombinant human PrP-(90–231) extending to 2.0 Å resolution has been observed and unlike observed monomeric NMR structures, the protein is shown to exist as a dimer (39). Three-dimensional domain swapping in this structure positions helix 1 at the dimer interface, with a rearranged disulfide linkage between α2 and the C-terminal α3 from the other polypeptide chain in the dimer.
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The low solubility and heterogeneity of the PrPSc species have impeded its structural analysis by NMR spectroscopy and x-ray crystallography. However, recent studies on two-dimensional crystals of PrP-(27–30), by electron crystallography and a higher resolution electron microscopy have improved our understanding of the structure of PrPSc (40, 41). The advanced PrPSc structural model contends that the sequence of PrP is compatible with a parallel left-handed β-helical fold that readily forms trimers. In agreement with these observations, our data demonstrate that various PrP species from monomers to tetramers, through to large aggregates, are present in brain homogenates from both uninfected and prion-infected human brains (Fig. 4, B and C). In uninfected human brains, PrP is mainly present in monomeric, dimeric, trimeric, and tetrameric forms, whereas a small amount of large PrP aggregates, also appears to be present (see Figs. 2, A and C, and 4C). By contrast, in prion-infected brains the levels of large PrP aggregates are dramatically increased, although the monomers and small oligomers are still detected (Figs. 2, B–D and 4B). Interestingly, our size exclusion chromatography reveals that oligomers at 4–5 monomers are predominately populated in the uninfected human brains. In view of the absence of significant PrP species between fractions 49 and 33 of sCJD, we assume that it may constitute a critical key oligomer for PrP aggregation. Although both the membrane proteins PrP and caveolin-1 show small amounts of large aggregates in the uninfected brains, in prion diseases the conversion of small oligomers to large aggregates seems to be unique to PrP, because this conversion does not occur with caveolin-1 (Fig. 3, A and B).

Studies on various species of recombinant PrP (rPrP) in vitro indicate that PrP possesses a highly flexible conformation. In aqueous solutions, rPrP can be folded into pH-dependent α-helical conformations, a thermodynamically more stable β-sheet, and various stable or transient intermediates (42). A kinetic stop-flow study demonstrated that prion protein folds by a three-state mechanism involving a monomeric intermediate (43). Moreover, using this approach, PrP mutations, linked with naturally occurring familial prion diseases, showed a pronounced stabilization of the folding intermediate (44). In addition to a β-oligomer and an amyloid fibril (45–48), two more polymeric transient intermediates have also been identified during fibrillogenesis of rPrP in vitro (49). Therefore, the tendency of PrP to form multiple non-native β-sheet-rich isoforms in vitro, as demonstrated in biophysical studies on rPrP, may represent a novel intrinsic feature of this protein.

A purified hamster PrPSc that displays an unexpectedly high β-sheet component under native conditions has in fact been reported (50). Also mammalian PrPSc has been shown to possess an intrinsic partial PK resistance (51). At a low concentration of PK, PrPSc was initially cleaved by PK to a 25–28-kDa intermediate fragment, prior to complete proteolysis. The cleavage site was proposed to lie between residues 112 and 144. It has been observed that normal human brain contains a substantial amount of an endogenously truncated PrP fragment, termed C1, migrating at ~18 kDa, whereas sCJD brains contain a relatively large amount of another endogenously truncated PrP fragment, termed C2, migrating at ~20 kDa (23). C1 has been shown to be sensitive to PK digestion and is believed to be a major product of PrPSc metabolism. C2 has been shown to be detergent-insoluble and is believed to be the “in vivo homologue” of PrP-(27–30), equivalent to that generated in vitro by PK digestion (23).

It was unexpected that the PrP20 would be detected preferentially by 1E4, but not 3F4, although the sequence of this PK-resistant fragment apparently also contains the 3F4 epitope. Of the three anti-PrP antibodies, including anti-C and 1E4, the affinity of 3F4 to the most common PK-resistant PrP core fragment PrP-(27–30) was actually the highest (Fig. 7, A–C). One possible interpretation is that 1E4 may be able to recognize a unique PrP species, in addition to those also detected by other anti-PrP antibodies. This is not unprecedented with anti-PrP antibodies. An antibody against human PrP-(95–110) (termed 8G8), that actually extends merely two more amino acids toward the N and C terminus of the 1E4 epitope, respectively, stained PrP-expressing baby hamster kidney cells with a brilliant cytoplasmic fluorescence (52). However, the number of positive cells was smaller when compared with the number of cells stained with other anti-PrP antibodies (52). This antibody was believed to only react with a subpopulation of the cells in culture. Furthermore, despite sharing a similar amino acid sequence within the corresponding region, only cattle, but not mouse and hamster PrP was observed to react with 8G8 (52).

The finding that there are small amounts of PrP aggregates and PK-resistant PrP conformers in the uninfected human brains, supports the hypothesis that potentially infectious PrP amyloid, so-called silent prions, may lie dormant in an apparently healthy individuals awaiting a change in the state of the host, or transmittal to a new more susceptible host (20, 13). How the PrP aggregates are generated in the uninfected human brains, and whether the formation of the aggregates is age-dependent remain to be determined. Several studies have demonstrated that PrP accumulated in the cytoplasm of cultured cells forms aggregates that are insoluble in non-ionic detergents and partially resistant to PK, under certain conditions, such as in a reducing environment, glycosylation, proteosomal inhibition, or expressing PrP without both N- and C-terminal signal peptides, albeit the absence of prion infection (53–57). Moreover, PrP has also been observed in the cytosol in subpopulations of neurons in the hippocampus, neocortex, and thalamus, but not in the cerebellum in uninfected wild-type mice (58). Whether the PrP aggregates and PK-resistant PrP fragments that we detected in the uninfected human brains might have been derived from the cytoplasm remains to be determined. Interestingly, based on the recent observation that the brains of bigenic mice are capable of clearing prions, Safar et al. (59) proposed that PrPSc is normally made at low levels and is continually cleared, and that PrPSc may have a function in cellular metabolism. Therefore, it is conceivable that under normal circumstances, although there is a small amount of PrPSc, the brain can maintain equilibrium between the formation and clearance of this PrPSc. The small amount of PrPSc does not induce a neurodegenerative disorder and thus can be considered to be in a silent state. However, significant increases in levels of the silent prions induced by infection or PrP mutation or unknown reasons may trigger prion diseases.
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Although the role of PrP amyloid fibrils in the pathogenesis of prion diseases remains debatable (60), a recent study on inoculation of transgenic mice overexpressing truncated PrP-(89–230) with recombinant PrP-(89–230) aggregates has clearly demonstrated that the presence of amyloid fibrils in the injected inocula is indeed critical for the initiation of prion disease (61). Furthermore, in scrapie-infected hamsters, it has recently been reported that the most infectious PrP aggregates were of sizes ranging from 300 to 600 kDa, whereas infectivity was virtually absent in oligomers of ≤5 PrP molecules (62). Therefore, initiation of prion formation and prion disease is clearly associated with PrP aggregation. Our finding that small amounts of PrP aggregates and PK-resistant PrP conformers are present in uninfected human brains might be an important starting point for mimicking an early stage of propagation and transmission of endogenous prions. Thus, it may be possible, for example, using the highly efficient protein misfolding cyclic amplification in vitro conversion system (16), with inoculation of transgenic mice expressing human PrP (26), to investigate these silent prions to learn if, or how, they might be amplified or if they are infectious.

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