Direct Evidence of Generation and Accumulation of β-Sheet-rich Prion Protein in Scrapie-infected Neuroblastoma Cells with Human IgG1 Antibody Specific for β-Form Prion Protein

We prepared β-sheet-rich recombinant full-length prion protein (β-form PrP) (Jackson, G. S., Hosszu, L. L., Power, A., Hill, A. F., Kenney, J., Saibil, H., Craven, C. J., Waltho, J. P., Clarke, A. R., and Collinge, J. (1999) Science 283, 1935–1937). Using this β-form PrP and a human single chain Fv-displaying phage library, we have established a human IgG1 antibody specific to β-form but not α-form PrP, PRB7 IgG. When prion-infected ScN2a cells were cultured with PRB7 IgG, they generated and accumulated PRB7-binding granules in the cytoplasm with time, consequently becoming apoptotic cells bearing very large PRB7-bound aggregates. The SAF32 antibody recognizing the N-terminal octarepeat region of full-length PrP stained distinct granules in these cells as determined by confocal laser microscopy observation. When the accumulation of proteinase K-resistant PrP was examined in prion-infected ScN2a cells cultured in the presence of PRB7 IgG or SAF32, it was strongly inhibited by SAF32 but not at all by PRB7 IgG. Thus, we demonstrated direct evidence of the generation and accumulation of β-sheet-rich PrP in ScN2a cells de novo. These results suggest first that PRB7-bound PrP is not responsible for the accumulation of β-form PrP aggregates, which are rather an end product resulting in the triggering of apoptotic cell death, and second that SAF32-bound PrP lacking the PRB7-recognizing β-form may represent so-called PrPSc with prion propagation activity. PRB7 is the first human antibody specific to β-form PrP and has become a powerful tool for the characterization of the biochemical nature of prion and its pathology. 

Prion diseases are fatal and transmissible neurodegenerative disorders that include Creutzfeldt-Jakob disease in humans and bovine spongiform encephalopathy in cattle. It has been proposed that a misfolded form of prion protein is responsible for the infectivity of prion disease, and the pathogenesis of prion disease involves a conformational change of prion protein (PrP) from PrPSc to PrPSc (1). PrPSc is a monomeric isoform, rich in α-helical structure, and sensitive to digestion by proteinase K (PK). In contrast, multimers are PrPSc characterized as PrPres by enhanced resistance toward PK digestion. Consequently, it is believed first that PrPSc might have a β-pleated sheet structure because all amyloids studied have been found to have this structure and second that this β-sheet–rich PrPSc...
β-Sheet-rich Prion Protein Aggregates in ScN2a Cells

binds specifically to PrPC, propagating its altered conformation via a templating mechanism, which is a prion activity. In other words, it was demonstrated that PrPSc, defined as PrP27–30, was generated and accumulated in prion-infected cells or brain. However, there is no direct evidence of whether prion or PrPSc has a β-sheet-rich structure, although there is suggestive evidence of FTIR using centrifugation-purified aggregates of prion-infected brain extracts (2, 3).

Antibodies to these proteins are powerful tools to clarify these questions. However, 1) the purification procedure of PrPSc was based on the centrifugation precipitation of molecular aggregates using PEG or sodium phosphotungstic acid, which does not necessarily guarantee the specificity to a prion (2, 4–7). 2) The specificity of an anti-PrPSc antibody was defined by the existence of PrP27–30 resulting from PK-treated prion-infected brain or cell extracts that are immunoprecipitated with tested antibodies. These experiments showed that PrP27–30 was resolved from PK-resistant PrP but do not directly indicate that PrP27–30 is generated from β-sheet-rich PrP; i.e. they rather suggest a generation of β-sheet-rich PrP by experimental procedures including the use of PK, denaturing agents, or detergents (8). 3) All antibodies established so far are either anti-PrPSc/PrPC or anti-PrP antibodies (9). More recently, mouse monoclonal IgG W261, which reacts exclusively with PrPSc but not PrPC, has been reported by cell fusion technology using spleen cells immunized with sodium phosphotungstic acid-precipitated PrPSc derived from prion-infected brain extracts (10). This study did not address the question of whether PrPSc was the β-form PrP or not.

In this study, using the conformation-defined recombinant PrPs and a human single chain Fv-displaying phage library, we have established two human IgG, PRB7 and PRB30, which are specific to the β-form but not the α-form of recombinant PrP of human, bovine, sheep, and mouse. Epitope mapping analysis showed that PRB7 IgG recognized residues 128–132 of the full-length prion protein. When prion-infected ScN2a cells were cultured in the presence of PRB7, apoptotic cells with numerous PRB7 binding signals including large aggregates were gradually generated during 4 days of culture. This finding is the first direct evidence of the generation and accumulation of β-sheet-rich prion protein in ScN2a cells. Interestingly, in these apoptotic cells, SAF32-staining granules were distinct from PRB7-binding aggregates, suggesting that SAF32-binding PrP does not have a PRB7-recognizing β-sheet structure, whereas PRB7-binding PrP may not have the N-terminal octarepeat region of PrP. After ScN2a cells were cultured in the presence of PRB7 or SAF32 for 3 days, PK-treated cell lysate was immunoblotted by 6D11 to examine the inhibitory effects of PRB7 IgG on the generation/accumulation of PrPSc. Surprisingly, PRB7 IgG had no influence, whereas SAF32 strongly inhibited the generation/accumulation of PrPSc.

Thus, this study reports the first establishment of a human IgG antibody recognizing β-form PrP but not α-form PrP and the use of this antibody to provide direct evidence of the de novo generation and conversion of β-sheet-rich PrP in prion-infected cells. PRB7 IgG can be a powerful tool to purify the β-form PrP generated de novo and demonstrate its biochemical basis and significance to elucidate structural evidence of prion infectivity and neurotoxicity.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—The pQE30 vector and Escherichia coli (M15) were obtained from Qiagen. The 6D11 antibody was purchased from Sigma. Recombinant anti-PrP Fab HuM-P, HuM-D18, HuM-D13, HuM-R72, and HuM-R1 were purchased from Inpro Biotechnology. GAHu/Fab/Bio was purchased from Nordic Immunology Inc. Horseradish peroxidase (HRP)-conjugated anti-goat mouse IgG, alkaline phosphatase-conjugated goat anti-mouse IgG, and goat anti-human IgG Fc-HRP were purchased from Jackson ImmunoResearch Laboratories. HisTrapTM HP, the anti-His tag antibody, and HitTrapTM Protein A HP were from GE Healthcare. The mouse anti-E tag monoclonal antibody, HRP-conjugated anti-E tag mAb, the anti-E tag antibody-Sepharose column, and the anti-M13 monoclonal antibody were purchased from Amersham Biosciences. The 3,3′,5,5′-tetramethylbenzidine solution was from Calbiochem. SAF32 was purchased from SPI Bio (Ann Arbor, MI). Control human IgG/κ was purchased from Bethyl Laboratories. Alkaline phosphatase-conjugated streptavidin and HRP-conjugated streptavidin were purchased from Vector Laboratories. The Dye Terminator Cycle Sequencing FS Ready Reaction kit was from Applied Biosystems. Nhel, BstApI, Apal, BsaW1, BbsI, and HindIII were obtained from New England Biolabs (Ipswich, MA). T4 DNA ligase was obtained from Takara Bio Inc. Opti-MEM, the SuperScriptTM First-Strand Synthesis System, pcDNA3.1TM(—) mycHisA, pcDNA3.1(—) Zeo, the FreeStyleTM MAX reagent, Annexin V-Alexa Fluor 568, Alexa Fluor 488-labeled anti-human IgG, Alexa Fluor 546-labeled anti-mouse IgG, and Alexa Fluor 488-succinimidyl ester were obtained from Invitrogen. Anti-β-actin antibody was purchased from Abcam. The labeling of antibody with Alexa Fluor 488 was performed according to the manufacturer’s instructions. DAPI was purchased from Cambrex Bio Science.

Expression Vector—PrP expression vectors were constructed as described (11). Briefly, DNA fragments corresponding to human PrP residues 23–231 were independently amplified by polymerase chain reaction (PCR) using forward primer (5′-ggc-gatcacaagagcgcagactcggag-3′) and back primer (5′-cca-agctttatacgctgatctcctctgtaata-3′) from genomic DNA of HEK293T cells. The fragments were ligated with BamHI and HindIII and inserted into a pQE30 vector bearing the His tag sequence. Bovine PrP(25–242), mouse PrP(23–231), or sheep PrP(25–234) expression vectors were constructed in a similar way.

Protein Expression and Purification—Constructed histidine-tagged recombinant full-length prion protein (rPrP; amino acids 23–231) was expressed in E. coli (M15) and purified by HisTrap HP as described (8). Briefly, fractions containing human rPrP were dialuted to a final concentration of 1 mg/ml using a 0.1 M Tris buffer (pH 8.0) containing 9 M urea and 100 mM β-mercaptoethanol. The dialyzed solution was diluted
3-fold with 0.1% (v/v) trifluoroacetic acid in water, loaded on a 10 × 150-mm WP300 C4 HPLC column (GL Sciences, Tokyo, Japan), and eluted using a gradient of 0.1% (v/v) trifluoroacetic acid in acetonitrile. Fractions containing oxidized PrP were lyophilized and stored at −20 °C as described (8).

Refolding—Refolding of rPrP was performed according to the methods of Jackson et al. (12). Briefly, to prepare α-form PrP, a stock solution of rPrP (1 mg/ml in 6 M GdnHCl) was dialyzed into a 10 mM sodium acetate, 10 mM Tris acetate buffer (pH 8.0). In the case of β-form rPrP, rPrP was dissolved in a 100 mM dithiothreitol (DTT), 6 M GdnHCl, 10 mM Tris acetate (pH 8.0) buffer for 16 h and then dialyzed with a 10 mM sodium acetate, 1 mM DTT (pH 4.0) buffer. A half-volume of the dialysis buffer was replaced with fresh buffer, and this procedure was repeated every 12 h for a total of 10 times. Refolded proteins were stored at 4 °C. The PrP concentration was determined using a spectrophotometer, Ultrospec 2000 (Amersham Biosciences), using a molar extinction coefficient $E_{280}$ of 56,650 M$^{-1}$ cm$^{-1}$.

Circular Dichroism Spectroscopy—Circular dichroism (CD) spectra were recorded on an SPI-3800 (Seiko Instruments, Chiba, Japan) immediately after dilution of the 5.4 mg/ml solution of the β-form PrP and displaying on a fresh mica surface.

PK Digestion—Recombinant PrP (100 μg/ml) was treated with the indicated concentration of PK for 1 h at 37 °C. ScN2a cell lysates were treated with PK (final concentration, 20 μg/ml) for 30 min at 37 °C, then PMSF at a final concentration of 2 mM was added, and the mixture was centrifuged at 15,000 rpm for 45 min at 4 °C. The pellet was dissolved in a 1× SDS-PAGE sample buffer and boiled for 10 min for immunoblotting analysis.

Immunoblotting—SDS-PAGE was performed as described (13, 14). Purified PRB7 IgG was separated by 10% SDS-PAGE, and the gel was stained with the silver stain II kit.

Immunoblotting was performed as described (13, 15). Briefly, samples were added to a 2× sample buffer, boiled for 10 min, separated on a 16.5% Tricine-polyacrylamide gel, and electrotransferred onto a PVDF membrane at 150 V for 90 min using a semidry electroblotter (Sartorius, Tokyo, Japan). The membrane was blocked with 5% skim milk in PBS overnight. Specific binding of the antibody to proteins was determined by incubating the membrane for at least 1 h with the 6D11 antibody (1:4000). HRP-conjugated goat anti-mouse IgG served as a secondary antibody (1:5000). Signal protein signals were visualized using ECL on a LAS-1000 image analyzer (Fuji Film, Tokyo, Japan). The Tricine gel was stained with the silver stain II kit.

Phage Libraries—The human single chain Fv (scFv) display library was constructed using cPANTAB 5E (4.5-kb) phagemid vector was used in this study as described (13, 15). The peptide-displaying phage libraries (Ph.D.-12) were purchased from New England Biolabs. The Ph.D.-12 library contains linear peptides composed of 12 random amino acids.

Biopanning—Biopanning was performed as described (13–15). To isolate the antibody phage specific to β-form PrP, the scFv phage library (1 × 10$^{12}$ pfu/50 μl) was preabsorbed with α-form PrP (5 μg) coating an immunotube under the conditions of a folding buffer (pH 8.0). One hour later, unbound phages were collected and added to a β-form PrP (5 μg)-coated immunotube under the conditions of a folding buffer (pH 4.0). After 1 h of incubation at room temperature, the unbound phages were discarded by washing the immunotubes seven times with folding buffer (pH 4) containing 0.1% Tween 20. The bound phages were then eluted with 0.1 M glycine–HCl (pH 2.2), immediately neutralized with 1 M Tris–HCl (pH 9.1), and amplified by infection with log phase E. coli strain TG1 cells. The phages and soluble scFv were prepared as described (13–15). To isolate peptide phage to PRB7 IgG, unrelated control human IgG/κ and PRB7 IgG were separately immobilized on polystyrene 96-well microplates (300 ng/50 μl/well). The Ph.D.-12 phage library (4 × 10$^{11}$ transforming units) was preabsorbed on a plate immobilized by unrelated control human IgG/κ. The phages were incubated at room temperature for 1 h with immobilized PRB7 IgG, and the binding phages were eluted with 0.1 M glycine–HCl (pH 2.2). The eluate was immediately neutralized with 1 M Tris–HCl (pH 9.1) and amplified by infecting ER2738.

Preparation of Aβ42 Conformers—Aβ42 conformers were prepared as described (14, 16). Synthetic Aβ42 peptides (purity 90–95% by mass spectrometer; Peptide Institute Inc., Osaka, Japan) were solubilized in 1,1,1,3,3,3-hexafluoro-2-propanol (Wako, Tokyo, Japan) at a concentration of 1 mg/ml (222 μM) and separated into aliquots in microcentrifuge tubes. Immediately prior to use, the 1,1,1,3,3,3-hexafluoro-2-propanol-treated Aβ42 was dissolved in dimethyl sulfoxide (Wako) to 4 mg/ml (888 μM) and diluted with a 20 mM phosphate buffer (pH 7.4) to a concentration of 40 μM.

ELISA—ELISA was performed as described (13–15). Briefly, a microtiter plate (Nunc, Denmark) was coated with refolded PrP (100 ng/50 μl/well) and anti-His tag mAb (80 ng/40 μl/well) for 1 h at room temperature. BSA (0.25%)/PBS was used as blocking buffer. BSA (0.25%)/PBS containing 0.1% Tween 20 was used as washing buffer. Anti-PrP Fab (HuM-P, HuM-D18, HuM-D13, HuM-R72, or HuM-R1; 1:500) (17–19) was detected by the anti-E tag antibody in combination with alkaline phosphatase-conjugated streptavidin. Soluble PRB7 scFv (200 ng/40 μl/well) was detected by the anti-E tag antibody in combination with alkaline phosphatase-conjugated goat anti-mouse IgG (1:1000). PRB7 IgG (0–1 μg/ml; 50 μl/well) was detected by goat anti-human Fc–HRP (1:2000; 50 μl/well). SAF32 (0.1 μg/ml; 50 μl/well) was detected by HRP-conjugated goat anti-mouse IgG (1:5000; 50 μl/well). Antibody phage clones (5 × 10$^{11}$ transforming units/40 μl) were incubated with the indicated refolded PrP (200 ng/40 μl) in a folding buffer (pH 4.0 or 8.0) and then captured on wells coated with an anti-His tag mAb. The bound phage was detected using a biotinylated anti-M13 mAb (1:1000) followed by the addition of alkaline phosphatase-conjugated streptavidin (1:1000). In the case of sandwich ELISA, PRB7 or control human IgG/κ (100 ng/25 μl) was incubated with human β-form PrP (100 ng/25 μl) in a pH 4.0 folding buffer.
buffer for 1 h and then added into wells precoated with an anti-PrP mAb, SAF32 or 6D11 (100 ng/50 μl). After treatment with a blocking and washing solution, the bound antibody was detected using an HRP-conjugated anti-human IgG (1:5000). In the case of the alkaline phosphatase development system, absorbance was measured at 405 nm during incubation with 50 μl of a p-nitrophenyl phosphate, 10% diethanolamine solution by use of a microplate reader (NJ-2300, PerkinElmer, Tokyo). In the case of the HRP development system, the plates were incubated with 50 μl of 3,3',5,5'-tetramethylbenzidine solution for 3 min at room temperature in the dark. After addition of 50 μl of 1 N HCl, absorbance was measured at 450 nm using the EnSpire 2300 Multilabel Reader (PerkinElmer Life Sciences).

To perform the epitope mapping of PRB7 IgG, a microtiter plate was coated with 50 ng/50 μl/H11032 EnSpire 2300 Multilabel Reader (PerkinElmer Life Sciences). Vlls forward primer (5'-H11032-AAAAAGCTAGCGATGGAAA-H11032'), Vlls back primer (5'-H11032-GAGGTGCAGCTGTTGCAG-3'), IGKC back primer (5'-AAGCTTCCAGACGAGGAGGAGAG-GAGGTGCAGCTGTTGCAG-3'), IGKC forward primer (5'-ACTGTGGCTGACACCATC-3'), and IGKC back primer (5'-AAGCTTCTAACACTCTCCCCCT-GTTG-3'). The IGKC1 fragment contains the Apal site at the 5' terminus and the HindIII site at the 3' terminus. The IGKC fragment contains the BbsI site at the 5' terminus and the HindIII site at the 3' terminus. The cDNA was prepared using the SuperScript First-Strand Synthesis System.

For the construction of the PRB7 IGHC1 expression vector, the Vhs fragment and the Vh fragment were digested with BstApI. These two fragments were ligated with T4 DNA ligase. The ligated sample was amplified by PCR using Vhs forward primer 2 (5'-AAAAATCTAGAGCTAGCGATGGCATG-3') and the Vh back primer. This amplified fragment and the IGKC fragment were digested with Apal, ligated, and amplified by PCR using Vhs forward primer 2 and the IGKC back primer. This amplified fragment was digested with Nhel and HindIII and inserted into a pcDNA3.1(−) mycHisA vector.

For the construction of the PRB7 IGHC1 expression vector, the Vlls fragment and the Vl fragment were digested with BsaWI, ligated with T4 DNA ligase, and amplified by PCR using the Vlls forward primer and the Vl back primer. This amplified fragment and the IGKC fragment were digested with BbsI, ligated, and amplified by PCR using the Vlls forward primer and the IGKC back primer. The amplified fragment was digested with Nhel and HindIII and inserted into a pcDNA3.1(−) Zeo vector.

Protein Expression and Purification of PRB7 IgG—FreeStyle 293F cells (Invitrogen; 1 × 10^6 cells/ml) were co-transfected with an equimolar mixture (final total DNA concentration, 1 μg/ml) of a heavy-chain expression vector and a light-chain expression vector with the FreeStyle MAX reagent (final concentration, 1 μl/ml) according to the manufacturer’s instructions (23). Transfections were performed in 500-μl cultures as described above. One week later, the supernatant was harvested from the culture by centrifugation. PRB7 IgG was purified using Hitrap Protein A HP according to the manufacturer’s instructions (13).

Computer Simulation of PrPs—The computer simulation was performed on the basis of the NMR structure of the human PrP (amino acids 124–227; Protein Data Bank code 1QMO) (24) using PyMOL software. Human IgG was simulated by use of Molecular Operating Environment™ (MOE, Version 2009.10) (supplemental Fig. 53).

Cell culture—ScN2a cells, N2a58 cells overexpressing mouse PrP (a genotype with codons 108L and 189T), FF32 cells, or N2aL1 cells were used. FF32 cells were cloned from F3 cells (an N2a58 cell line infected with a prion Fukuoka-1 strain derived from mouse brain infected with a brain of a Gerstmann-Straussler-Scheinker syndrome patient) or N2aL1 cells (an N2a58 cell line infected with the 22L strain derived from mouse brain infected with scrapie) as described previously (25, 26). All cells were cultured in Opti-MEM containing 10% fetal bovine serum and penicillin-streptomycin at 37 °C in 5% CO₂. Cells (5 × 10^6 cells/well in a 24-well plate) were cultured in the
RESULTS

Characterization of Recombinant Human Full-length PrP—
PrP was prepared using recombinant prion protein as described (8). CD analysis showed the typical characteristics of the α-form of PrP with a structure rich in α-helical content and of the β-form with a β-sheet rich structure, respectively (Fig. 1A) (31). Atomic force microscopy of the β-form of PrP displayed a round shaped particle, showing the formation of globular structures with an apparent diameter (horizontal) of ~47 nm and an apparent height of ~1.6 nm (Fig. 1B). α-Form PrP was digested completely with 2 μg/ml PK, whereas β-form PrP showed resistant fragments at 8 μg/ml PK treatment (Fig. 1C). D18 antibody recognizes the epitope 133–157 of α-form PrP but not β-form PrP (17–19). In accordance with this finding, D18 did not bind to this β-form PrP. D13 (epitope 96–106 of PrP), R72 (epitope 152–163 of PrP), or the P antibody (epitope 96–105 of PrP) did not distinguish the structural difference between the α-form and the β-form of PrP (Fig. 1D) (17–19).

Biopanning against β-Form of PrP—The immunotube for β-form PrP because PrP fraction 2 exhibited a typical β-sheet structure in CD analysis (Fig. 1B) and 12.6 kDa (β-form PrP fraction 1) at an elution time of 7.5 (β-form PrP fraction 1) and 12.6 min (β-form PrP fraction 2) in a running buffer at pH 4.0. For the β-form of PrP, the sample was resolved as two peaks at an elution time of 7.5 (β-form PrP fraction 1) and 12.6 min (β-form PrP fraction 2) in a running buffer at pH 4.0. (20 mM sodium acetate, 200 mM sodium chloride, 1 mM urea, 0.02% azide). For the β-form of PrP, the sample was resolved as two peaks at an elution time of 7.5 (β-form PrP fraction 1) and 12.6 min (β-form PrP fraction 2) in a running buffer at pH 4.0. (20 mM sodium acetate, 200 mM sodium chloride, 1 mM urea, 0.02% azide) (Fig. 4A). α-Form PrP fraction 1 exhibited a typical α-helical spectrum, whereas β-form PrP fraction 1 and β-form PrP fraction 2 exhibited a typical β-sheet structure in CD analysis (Fig. 4B).

To test the binding activity of these fractions to PRB7 and PRB30, each fraction was immobilized on microtiter plates. As shown in Fig. 4C, PRB7 or PRB30 bound to β-form PrP fraction 1 as well as β-form PrP fraction 2. Both scFv showed no binding to α-form PrP, amyloid β42 monomer peptide, IL-18, or BSA. In contrast, the authentic mouse anti-PrP monoclonal antibody SAF32 bound to every PrP fraction irrespective of their conformations. Its binding epitope is located at the N-terminal octapeptide region of the α-form of PrP (27). Unrelated scFv (RE51) gave no binding reactions to any coated proteins.

PRB7 IgG and Its Binding Specificity—IgG form of PRB7 scFv was constructed to endow PRB7 scFv with molecular stability. PRB7 IgG purified with a Protein A column was analyzed by
SDS–PAGE, which indicated that the IgG form associated with a γ- and κ-chain with a disulfide bond (Fig. 5A). The PRB7 IgG showed an identical binding specificity to PRB7 scFv with specific binding to β-form PrP but not α-form PrP, whereas SAF32 bound equally to β-form PrP and α-form PrP (Fig. 5B). Furthermore, PRB7 IgG showed no binding activity to Aβ42 soluble form, prefibril oligomers, or fibril forms (Fig. 5B).

**Epitope Mapping of PBR7 IgG**—Epitope mapping was performed using a linear peptide-displaying phage library (Ph.D.-12) and a circular peptide-displaying phage library (Ph.D.-C7C). PRB7 IgG-binding clones (pepPRB7-14) were isolated from Ph.D.-12 among 70 clones tested, whereas no clone was selected from Ph.D.-C7C. The binding activity of PBR7 IgG to the pepPRB7-14 clone is shown in Fig. 6A. The amino acid sequence homology search using ClustalW version 3.1 indicated that the pepPRB7-14 peptide motif had weak homology to 124–136, particularly 128–132 of the human full-length prion protein, suggesting that PRB7 IgG recognizes this epitope (Fig. 6B). The 124–136 region is depicted in red in the PyMOL display of the NMR structure of the α-form of PrP (24).
PRB7 IgG Recognizes Common β-Conformation of PrP
Shared by Human, Bovine, Sheep, and Mouse—Recombinant PrP of human, bovine, mouse, or sheep was folded according to the protocol of Jackson et al. (12). The CD spectrum of each PrP preparation is shown in Fig. 7A. When the binding activity of PRB7 IgG to these PrPs was examined by ELISA, PRB7 IgG exclusively
bound to the β-form of PrP but not the α-form of PrP (Fig. 7B, left panel). This reactivity is shared by human, bovine, sheep, and mouse PrP. SAF32 strongly recognized both forms of every species without any discrimination (Fig. 7B, right panel).

β-Form PrPs Are Generated and Accumulate de Novo in Prion-infected ScN2a Cells—PRB7 recognizes the fine structure of the conformation of PrP and does not stain ScN2a cells fixed with paraformaldehyde. Furthermore, PRB7 IgG hardly stained...
cell surfaces of non-fixed ScN2a cells, suggesting scarce existence of β-form PrP on the cell surface (supplemental Fig. S6). Therefore, to examine the generation of β-form PrP in prion-infected cells, prion-infected ScN2a cells were cultured in the presence of PRB7 IgG or SAF32. Three days later, cells were fixed with paraformaldehyde. After permeation with saponin, cells were stained with Alexa Fluor 488-anti-human IgG to detect the complex of PRB7 IgG-PrP or SAF32-PrP (Fig. 8A). Control cells, N2a58 non-infected cells, or ScN2a cells in the presence of normal IgG were not stained with this protocol. As the binding of normal IgG to N2a58 cells or ScN2a cells was not observed by confocal microscopy or flow cytometry analysis, N2a58 cells or ScN2a cells were found to be Fcγ receptor-negative. In contrast, PRB7 IgG or SAF32 stained the cytoplasm of ScN2a but not N2a58 in a distinct image. Three staining patterns of cytoplasm were observed in ScN2a incubated with PRB7 IgG, i.e. cells with varying numbers of small granules, cells with very large aggregates in the cytoplasm, and cells with no staining. SAF32 stained cell surface PrP of ScN2a and N2a58 cells. When detection sensitivity of confocal laser microscopy was increased, SAF32 was detected by Alexa Fluor 546-labeled anti-mouse IgG in the cytoplasm region below the cell surface.

FIGURE 5. Characterization of PRB7 IgG antibody. A, SDS-PAGE of PRB7 IgG. Protein A column-purified PRB7 IgG (300 ng) was separated by 10% SDS-PAGE under reducing or non-reducing condition and detected by silver staining. B, PrP conformers (50 ng/50 μl/well) were detected by PRB7 IgG or SAF32 (0.5 μg/ml). Aβ42 conformers (50 ng/50 μl/well) were tested for binding reactivity to PRB7 IgG or 6E10 (mouse anti-Aβ42 antibody) as described under “Experimental Procedures.” 2ME, 2-mercaptoethanol. Data represent the mean values ± S.D.
membrane of ScN2a. When these cells were fixed with methanol and stained with anti-β/HT9252-actin or anti-β/HT9252-tubulin antibody, the PRB7 IgG-bound aggregates were evident in ScN2a cells cultured with PRB7 IgG (Fig. 8B). The details of the PRB7-staining features of ScN2a cells cultured with PRB7 IgG are investigated in Fig. 9. About 4000 cells were classified on the basis of the number of stained granules. Cells without granules amounted to 71%, and those with one granule amounted to 16%. Cells with more than 10 granules amounted to 0.6%. Among these cells, 0.4% of cells with very large aggregates in the cytoplasm were particularly conspicuous. Although ScN2a cells with enormous aggregates in the entire cytoplasm were stained with anti-β-actin (Fig. 8B), these cells were analyzed for the existence of nuclei over the perpendicular axis using serial images taken by confocal microscopy, and traces of nuclei were detected by DAPI (Fig. 10A, white arrow). Annexin V weakly

FIGURE 6. Epitope mapping of PRB7 IgG. A, peptide phage clones (50 µl/well of 1 × 10^11 virions/ml) were incubated with PRB7 IgG (50 ng/50 µl/well). The bound peptide phage clones were detected using a biotinylated anti-M13 monoclonal antibody (1:2000) followed by HRP-conjugated streptavidin (1:2000). The pepPRB7-14 was selected from a Ph.D.-12 peptide phage library. Data represent the mean values ± S.D. B, amino acid sequence homology between the full-length human PrP and pepPRB7-14 motif (red) was searched by ClustalW. The matching region (amino acids 124–135) is depicted in the red box. The secondary structures are indicated by explanatory marks as shown in the black box. C, computer simulation model of human α-form PrP (Protein Data Bank code 1QM0). The matching region (amino acids 124–135) containing a highly homologous region (amino acids 128–132) is depicted as a red ribbon. PRB7 IgG does not recognize this structure of α-form PrP.
stained these cells with enormous aggregates, indicating that these cells had undergone apoptosis, whereas Annexin V did not stain cells that were strongly DAPI-positive (Fig. 10A). When cells with enormous aggregates were stained with SAF32 in addition to PRB7, however, this staining image observed from the perpendicular axis was not completely merged in PRB7- and SAF32-staining PrP (Fig. 10B, white arrow). A magnified image of the B region of Fig. 10B indicated that PRB7 IgG stained very large tangled fibrillary aggregates, whereas SAF32 appeared to independently stain distinctive tiny granules dispersed in the entire cytoplasm (Fig. 10C, right panel). Similarly, in the case of ScN2a cells with clear DAPI-positive nuclei and numerous small PRB7 IgG-bound granules, SAF32 stained another part of the cytoplasm distinct from PRB7 IgG (Fig. 10C, left panel). These cells with a number of tiny PRB7-positive granules were clearly stained with Annexin V, indicating that they had undergone apoptosis (Fig. 10D). These results suggested that PRB7 IgG-bound granules were antibody complexes with N-terminal region-deleted PrP. To confirm whether PRB7 IgG-bound complexes were composed of PrP, ScN2a cells cultured with Alexa Fluor 488-labeled PRB7 IgG were denatured with 6 M GdnHCl and stained with another anti-PrP antibody, 6D11, which recognizes the epitope located at 93–109 of PrP (32). PrP is sandwiched with PRB7 IgG and 6D11 in ELISA (supplemental Fig. S7). As shown in Fig. 10E, SAF32 or 6D11 did not visualize tiny aggregates in the cytoplasm of non-denatured ScN2a cells, whereas PRB7 IgG stained numerous tiny aggregates in the cytoplasm (Fig. 10E).
panels 3 and 5). PRB7 IgG showed a distinct image in the same cells (Fig. 10E, panel 4) where two kinds of staining images were observed, i.e. non-merged and merged aggregates with PRB7 IgG and 6D11 (Fig. 10E, panels 6 and 8). Fig. 10E, panel 7, shows a three-dimensional image of Fig. 10E, panel 6. These results suggested that there were not only PRB7 IgG-bound aggregates that had lost SAF32 and 6D11 epitopes but also 6D11 epitope-retaining aggregates bound with PRB7 IgG.

**PRB7 IgG Showed No Inhibitory Activity on Accumulation of PrPres in ScN2a Cells**—ScN2a cells were cultured in the absence or presence of PRB7 IgG, SAF32, control human IgG/κ, or control mouse IgG (5 μg/ml) in 10% FBS, Opti-MEM. Three days later, cells were fixed with paraformaldehyde and permeated for staining with Alexa Fluor 488-labeled anti-human IgG (green), Alexa Fluor 546-labeled anti-mouse IgG (red), or DAPI (blue) as described under “Experimental Procedures.” Each box indicates a representative staining pattern. Box 1, Box 2, and Box 3 show large/many granules, a few tiny granules, or no granules stained with PRB7 IgG, respectively. B, ScN2a cells cultured in the presence of PRB7 IgG or control human IgG/κ (hIgG) (green) as described in A were fixed with methanol and stained with anti-β-actin antibody (red), anti-β-tubulin antibody (red), or DAPI (blue) as described under “Experimental Procedures.”

**DISCUSSION**

A number of studies showed that prions generate and accumulate PrPres, which shows resistance to PK degradation. It is postulated that PrPres converges PrPSc to PrPSc by its templating activity. PrPSc was purified as a multimer or aggregates. The protease-resistant core of PrPSc, designated PrP27–30, polymerizes into an amyloid. Many purified amyloids have been found to have a β-pleated sheet structure. From these findings, it is believed that prion or PrPSc may have a β-sheet-rich structure (3, 5).

Is PrPSc a β-form PrP? Antibodies should be a powerful tool to solve this question. Despite the insufficient biochemical characterization of PrPSc, many attempts have been made to establish antibodies with fine specificity using mice immunized

**FIGURE 8. β-Form PrPs are generated and accumulate de novo in prion-infected ScN2a cells.** A, ScN2a or N2a58 cells were cultured in the absence or presence of PRB7 IgG, SAF32, control human IgG/κ, or control mouse IgG (hIgG) (green) as described in A were fixed with methanol and stained with anti-β-actin antibody (red), anti-β-tubulin antibody (red), or DAPI (blue) as described under “Experimental Procedures.”
with partially purified PrPSc from prion-infected cells or brain extracts (6, 10, 33–42). However, most antibodies were specific to PrPSc or cross-reactive to both PrPSc and PrPSc but not monospecific to PrPSc, although more recently a PrPSc-specific murine IgG1 antibody, W261, has been established (10). However, even in this situation, PrP conformation-specific antibodies have not been developed.

In this study, using conformation-defined recombinant β-sheet-rich PrP and an antibody-displaying phage library, we established a β-sheet-rich PrP-specific human IgG1 antibody, PRB7 IgG. This antibody does not recognize generic oligomer or aggregate forms of unrelated proteins in accordance with the binding activity of PRB7 or PRB30 scFv. PRB7 IgG is the first human IgG antibody specifically binding to β-sheet-rich PrP monomer and oligomers but not α-form PrP. It is suggested that PRB7 IgG recognizes the epitope 128–132 of β-sheet-rich human PrP. PRB7 IgG is equally cross-reactive to the β-sheet-rich of bovine, sheep, and mouse PrP.

As PRB7 IgG does not recognize paraformaldehyde-fixed or denatured PrP molecules, we simply observed the ScN2a cells cultured with PRB7 IgG. We have demonstrated here that prion-infected ScN2a cells specifically internalized PRB7 IgG and accumulated the PrP granules bound with PRB7 IgG in the cytoplasm (Fig. 8). This is the first direct demonstration that β-sheet-rich PrP was generated and accumulated in prion-infected cells in the physiological condition. Because neither ScN2a nor N2a58 cells were stained with normal IgG as assessed by confocal microscopy or flow cytometry, these cells were Fcγ receptor-negative. Nevertheless, ScN2a cells internalized PRB7 IgG, suggesting that complexes of β-sheet-rich PrP targeted by PRB7 IgG

| Number of granular PrP | PRB7 IgG & DAPI | DIC | Merge | Number of cells / Number of total cells (%) |
|------------------------|-----------------|-----|-------|---------------------------------------------|
| 0                      |                 |     |       | 3008 / 4203 (71.6%)                          |
| 1                      |                 |     |       | 686 / 4203 (16.3%)                           |
| 2                      |                 |     |       | 226 / 4203 (5.4%)                            |
| 3                      |                 |     |       | 97 / 4203 (2.3%)                             |
| 4                      |                 |     |       | 61 / 4203 (1.5%)                             |
| 5                      |                 |     |       | 35 / 4203 (0.8%)                             |
| 6                      |                 |     |       | 21 / 4203 (0.5%)                             |
| 7                      |                 |     |       | 11 / 4203 (0.3%)                             |
| 8                      |                 |     |       | 5 / 4203 (0.1%)                              |
| 9                      |                 |     |       | 4 / 4203 (0.1%)                              |
| 10                     |                 |     |       | 6 / 4203 (0.1%)                              |
| >10                    |                 |     |       | 26 / 4203 (0.6%)                             |
| non-nuclear cell       |                 |     |       | 17 / 4203 (0.4%)                             |

**FIGURE 9.** Distribution pattern of PRB7 IgG-positive granules in ScN2a cells cultured with PRB7 IgG. Cells were stained with PRB7 IgG or DAPI as described in Fig. 8. A total of 4203 cells were classified by the number of granules in the cytoplasm (positive cells/total cells observed). DIC, differential interference contrast.

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**β-Sheet-rich Prion Protein Aggregates in ScN2a Cells**

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A

B

C

D

E

| None | GdnHCl (+) |
|------|------------|
| Merge (DAPI, SAF32) | Merge (DAPI, PRB7, 6D11) |
| PRB7 IgG | PRB7 IgG |
| GdnHCl (+) | GdnHCl (+) |

6D11 | Merge (4, 5) | Merge (4, 5) | Merge (DAPI, PRB7, 6D11) |
localizing on the cell surface were endocytosed into the cytoplasm (29). If this β-form PrP corresponds to PrPSc, this observation is in accordance with the finding that PrPSc was generated on a lipid raft on the cell surface (43). When ScN2a cells were cultured with PRB7 IgG, they gradually generated an increasing number of tiny PRB7-binding granules in the cytoplasm. Among them, about 0.4% of cells had large aggregates in the cytoplasm. Vertically severed images of these cells show traces of nuclei in cells that were weakly stained with Annexin V, indicating that they had undergone apoptosis (Fig. 10A). These findings suggested that an enormous aggregate resulted from the fusion of many tiny β-form granules primarily endocytosed from the cell surface but was not the result of β-form PrP propagation in the cytoplasm.

SAF32 bound tiny granules dispersed in the cytoplasm that were distinct from large aggregates stained with PRB7 IgG (Fig. 10C). These results suggested first that PRB7-positive β-form PrP–PSs lost or hid the N-terminal octarepeat region to which SAF32 bound and second that granules stained with SAF32 were not generated from β-form PrP.

It was suggested that PrPSc–PrPSc conversion, which is physiologically prevented by an energy barrier, may be a spontaneous stochastic event favored by mutations in the PRNP gene or acquired by infection with exogenous PrPSc. Accordingly, it is conceivable that GdnHCl treatment of cells additively induces the conformational conversion of PrP, leading to the formation of aggregates in experiments that immunohistologically attempted to show granules of PrPSc in the cytoplasm (8). It is noted that when ScN2a cells were stained with SAF32 or 6D11 with a denaturing pretreatment by 6 M GdnHCl numerous tiny PrP granules became visible in the cytoplasm, and their images were quite distinct from those of PRB7-staining aggregates. Our results indicated that apoptotic cells showed two distinct types of PrP granules in the cytoplasm: one was SAF32-negative and PRB7 IgG-positive granules (N-terminal region-deleted; β-form PrP), and the other was PRB7 IgG-negative and SAF32-positive granules (full-length PrP lacking a β-sheet-rich conformation) (Fig. 10E). Related studies have reported that PK-sensitive PrPSc was contained in prion aggregates (44) and more recently that there is heterogeneity in PrPSc conformers including small soluble PrP aggregates (6). Regarding the requirement of full-length PrP for prion activity, the answer is not consistent: i.e. Prusiner et al. (50) reported that the purified PK-digested PrP27–30 was not infectious, whereas Anaya et al. (45) have recently reported that small infectious PrPRES aggregates were recovered in the absence of strong in vitro denaturing treatments from prion-infected cultured cells. On the other hand, using an anti-aggregated PrP IgM antibody, 15B3, Biasini et al. (6, 47) reported the successful purification of pathological full-length prion aggregates, and Cronier et al. (46) found that PK-sensitive PrPSc was involved in prion aggregates. Our findings in Fig. 10 suggest that PrP composed of tiny aggregates visualized with SAF32 or 6D11 after 6 M GdnHCl treatment of cells may be a full-length PrP with α-conformation.

To examine the influence of PRB7 IgG on the templating activity of β-form PrP to accumulate PrPSc, ScN2a cells were cultured in the presence of PRB7 IgG or SAF32, and their PK-treated cell lysates were immunoblotted with 6D11 to evaluate the amount of PrPRES. Surprisingly, PRB7 IgG had no effect.
whereas SAF32 strongly inhibited the generation/accumulation of PrPSc. Similar results have been reported in which a PrPSc-specific antibody had no prion-clearing effect, whereas the PrPSc-specific antibody showed marked clearing activity (10). Because the mimotope of PRB7 is located at 128–132, if this β-pleated sheet forms an interface for prion aggregation PRB7 IgG may block the assembly of PrP aggregates because the binding IgG is much larger than the target molecule (PrP). We examined this activity using FF32 cells or N2aL1 cells and obtained the same results as for ScN2a cells (data not shown). In this respect, our result suggested that, in the prion conversion reaction, PRB7 IgG-recognizing β-form PrP did not play a role in driving the conversion of the PrP conformation and appears rather to be an end product that loses prion activity and accumulates in the cytoplasm. This end product may trigger cell cytotoxicity via the mitochondrial machinery (42). This is in concert with the result that β-form recombinant PrP does not work as a template for prion amplification in protein misfolding cyclic amplification (41, 48). It is conceivable that PRB7-negative, SAF32-positive granules as a full-length, non-β-form PrP might be responsible for prion propagation (Fig. 10, C and E). Our results may be in agreement with the finding that prion propagation and toxicity in vivo occur in two distinct mechanistic phases (42). Thus, we have not obtained evidence to determine whether PRB7-recognizing β-form PrP represents the so-called PrPSc that has both conversion activity and cell cytotoxicity. Further investigation is needed to determine whether β-form PrP affinity-purified with PRB7 shows prion activity such as the infectious and conversion/accumulation activity of the prion.

Although a number of studies illuminated the accumulation of PrPres in prion-infected cells or brain extracts, there is little literature giving much attention to the phenomenon in which most prion-infected ScN2a cells vigorously proliferate to survive in culture (49). The low frequency of PRB7 IgG-positive granules in ScN2a cells suggests the stochastic nature of the conversion of α-form to β-form PrP. The spongelike degeneration of prion-infected brain may reflect this feature.

In conclusion, we report the establishment of PRB7 IgG, which is the first human antibody discriminating the β-form from the α-form of PrP in the physiological condition. Using this antibody, we have demonstrated direct evidence of the generation and accumulation of β-form PrP in prion-infected ScN2a cells. It can be used to purify β-form PrP molecules without the need for protease digestion and may be useful to demonstrate the biochemical basis of the relationship of β-form PrP to PrPSc and to elucidate the structure-based evidence for prion infectivity and neurotoxicity.

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