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Conversion of Bacterially Expressed Recombinant Prion Protein

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Abstract

The infectivity associated with prion disease sets it apart from a large group of late-onset neurodegenerative disorders that shares the characteristics of protein aggregation and neurodegeneration. The unconventional infectious agent, PrP^{Sc}, is an aberrantly folded form of the normal prion protein (PrP^C) and the PrP^C-to-PrP^{Sc} conversion is a critical pathogenic step in prion disease. Using the Protein Misfolding Cyclic Amplification technique, we converted folded bacterially expressed recombinant PrP into a Proteinase K-resistant and aggregated conformation (rPrP-res) in the presence of anionic lipid and RNA molecules. Moreover, high prion infectivity was demonstrated by intracerebral inoculation of rPrP-res into wild-type mice, which caused prion disease with a short incubation period. The establishment of the *in vitro* recombinant PrP conversion assay makes it feasible for us to explore the molecular basis behind the intriguing properties associated with prion infectivity.

Keywords

Prion; recombinant protein; *in vitro* conversion; infectivity; neurodegeneration

1. Introduction

Transmissible Spongiform Encephalopathies, the prion diseases, are a group of fatal neurodegenerative disorders that occur in a wide variety of mammals (1–3). The conversion of soluble prion protein (PrP^C) to the aggregated pathogenic conformer (PrP^{Sc}) is closely associated with pathogenesis of prion disease (4–6). Extensive efforts have been exerted to understand the mechanism of PrP conversion. In an *in vitro* cell-free system, purified PrP^C is converted to a PrP^{Sc}-like Proteinase K (PK)-resistant conformation with low efficiency (7). More efficient PrP conversion has been achieved by subjecting a mixture of brain homogenates prepared from healthy and prion-infected animals to multiple cycles of sonication and incubation, a technique named as Protein Misfolding Cyclic Amplification (PMCA) (8). Notably, both PrP^{Sc} conformer and prion infectivity are amplified via PMCA (9). Higher conversion efficiency associated with crude brain homogenates suggests the involvement of other factors in PrP conversion. Indeed, lipid-PrP interaction was found to be essential for PrP conversion in the cell-free conversion assay (10). Polyanionic molecules, such as RNA, have been shown to facilitate the *in vitro* amplification of PrP^{Sc} during PMCA (11,12). Furthermore, *de novo* generation of an infectious prion has been achieved using

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PrP^C purified from wild-type hamster brain together with co-purified lipid molecules and synthetic polyanionic molecules (13). All these findings support a critical role of facilitating factors in PrP conversion.

We previously showed that bacterially expressed recombinant mouse PrP (rPrP) undergoes conformational changes and gains PrP^{Sc}-like PK-resistance after interacting with anionic lipids (14). Recently, we tested the conversion of rPrP in the presence of anionic lipid and RNA molecules using the PMCA approach. A misfolded PK-resistant rPrP form (rPrP-res) was generated after multiple rounds of PMCA (15). The newly formed rPrP-res molecules recapitulate the distinct properties of naturally occurring prions: insolubility in mild detergent, partial resistance to PK digestion and capability of propagation by converting rPrP *in vitro* and endogenous PrP^C in cultured cells. Intracerebral injection of rPrP-res in wild-type mice caused extensive neurodegeneration and the accumulation of aggregated PrP in the brain, which are the classical neuropathological changes of prion disease (15).

2. Method description

2.1. Expression and purification of recombinant mouse prion protein (rPrP)

Recombinant prion proteins expressed in *E. Coli* have been used extensively in studies analyzing the three dimensional structure of PrP (16–20) and PrP conversion (21–25). The structure of rPrP was validated by a study showing that the three-dimensional structures of rPrP was essentially identical to that of PrP^C isolated from healthy calf brains (26). The rPrP used in our experiment was purified using the same protocol that has been shown to fold rPrP into correct tertiary structure (14,27).

2.1.1. Constructing rPrP expressing vector—The DNA sequence of mouse PrP23-230 was amplified by PCR using the following primers (Integrated DNA Technologies, Inc.).

5' AAAGGATCCAAAAAGCGGCCAAAGCCTGGA 3'

5' CCCAAGCTTAGGATCTTCTCCCGTCGTAATA 3'

The amplified DNA fragments were purified using QIAquick PCR Purification Kit (Qiagen, USA) and digested with restriction endonucleases, BamHI and HindIII (New England Biolabs, Inc.). Digested DNA was subjected to agarose (Shelton Scientific-IBI) electrophoresis and recovered using QIAquick Gel Extraction Kit (Qiagen, USA). Protein expressing vector pPROEX-HTb (Invitrogen) was digested with BamHI and HindIII and recovered from agarose gel using QIAquick Gel Extraction Kit. The digested PrP23-230 DNA fragments and pPROEX-HTb vector were ligated using T4 DNA ligase (New England Biolabs, Inc.) at 14°C overnight and the reaction mixture was used to transform *E. Coli* DH5α competent cells (Invitrogen). Plasmids were isolated using QIAprep Spin Miniprep Kit (Qiagen, USA) and verified by DNA sequencing. The plasmid with correct PrP23-230 insert was used as template for a site-direct mutagenesis reaction using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and the following primers (Integrated DNA Technologies, Inc.).

5' GAAAACCTGTATTTTCAGGGCAAAAAGCGGCCAAAGCCTGG 3'

5' CCAGGCTTTGGCCGCTTTTTCGCCCTGAAAATACAAGTTTTC 3'

The mutagenesis eliminated the 3 amino acids immediately before mouse PrP23-230 coding sequence and created a construct, pPROEX-HTb-moPrP23-230, expressing recombinant mouse PrP23-230 with one extra amino acid residue, glycine, at the very N-terminus after

removing the histidine tag by the tobacco etch virus (TEV) protease. The construct was verified by DNA sequencing.

2.1.2. Expression of rPrP—The LB liquid medium (Difco LB Broth, Miller (Luria-Bertani), BD Biosciences) with 100 µg/ml Ampicillin and 34 µg/ml Chloramphenicol was used for bacterial culture, and all cultures were carried out in a New Brunswick incubator at 37°C with 250 rpm agitation. A single colony of pPROEX-HTb-moPrP23-230 transformed *E. Coli* BL21 (DE3) cells was first cultured overnight in 5 ml LB medium. Next day, 1 ml overnight culture was inoculated into 100 ml LB medium and cultured overnight. Next morning, 30 ml overnight culture of *E. Coli* cells were inoculated into 1 liter of LB medium and cultured until OD₅₉₀=0.5–0.6. Then 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Invitrogen) was added and the culture was continued for 5 more hours.

2.1.3. Purification of rPrP—Purification of rPrP was performed according to a previously reported protocol (27) with a few adaptations.

Buffers used in purification are listed below.

Buffer A: 10 mM Tris-HCl, 100 mM NaPO₄, pH 8.0

Buffer B: 6 M GdnHCl, 10 mM Tris-HCl, 100 mM NaPO₄, 10 mM βME, pH 8.0

Buffer C: 10 mM Tris-HCl, 100 mM NaPO₄, 50 mM Imidazole, pH 8.0

Buffer D: 10 mM Tris-HCl, 100 mM NaPO₄, 500 mM Imidazole, pH 5.8

Buffer E: 10 mM NaPO₄, pH 5.8

Buffer F: 10 mM NaPO₄, pH 6.5

10 × TEV reaction buffer: 0.5 M Tris-HCl (pH 8.0), 5 mM EDTA

Induced *E. Coli* cells were harvested (5,000 × g, 10 minutes, 4°C) and resuspended in 75 ml of buffer A. Resuspended cells were lysed through four 2-minute rounds of sonication on ice with output = 6 and duty cycle = 80% (Branson Sonifier 450). Cell lysates were incubated on ice for 10 minutes between each round of sonication. Inclusion bodies in cell lysates were collected (15,000 × g, 30 minutes, 4°C), resuspended in 75 ml of buffer B, and sonicated on ice till completely solubilized (4 rounds of 4-minute sonication, output=6, duty cycle=80%, Branson Sonifier 450, 10 minute break between each round). After centrifugation (15,000 × g, 30 minutes, 4°C), the supernatant was mixed with 30 ml of Nickel-nitrilotriacetic acid (Ni-NTA superflow) agarose resin (Qiagen, USA) (pre-equilibrated with buffer B) and then stirred for 30 minutes at room temperature. The resin was then packed into a glass chromatography column (Bio-Rad) and washed with 120 ml of buffer B. Afterwards, 200 ml gradient of buffer B to buffer A was applied to resin in column (2 ml/minute), which was followed by a wash with 75 ml buffer C to remove the impurities devoid of the histidine tag. The histidine tagged rPrP was eluted with Buffer D. The eluted fractions with OD₂₈₀ reading equal or above 0.1 were combined, added to SnakeSkin Pleated Dialysis Tubing (10,000 MWCO, Pierce), and dialyzed for 1.5 hours against buffer E (Buffer E was changed every 30 minutes) and another 1.5 hours against deionized water (Deionized water was changed every 30 minutes). For TEV cleavage of the histidine tag, 10 × TEV buffer and TEV protease were added, successively, drop by drop into dialyzed protein solution that was stirred continuously. The mixture was centrifuged (5,000 × g, 10 minutes, 4°C) and the supernatant was incubated at 30°C until all histidine tags were removed from rPrP (Usually, an overnight digestion completes the cleavage. The efficiency of cleavage was verified by silver staining of SDS-PAGE gel). Then the digestion mixture was loaded into a 30 ml CM sepharose column (CCF100, Sigma) (pre-equilibrated with buffer F) and eluted with 200 ml gradient of 0–500 mM NaCl (2 ml/minute). Eluted fractions with OD₂₈₀ reading equal or

above 0.1 were combined and dialyzed against deionized water for 3 hours (deionized water was changed every 30 minutes) using SnakeSkin Pleated Dialysis Tubing (10,000 MWCO, Pierce). Dialyzed rPrP in deionized water was kept at -80°C . The purity of recombinant protein was monitored by silver staining on SDS-PAGE gel (Fig. 1). The concentration of purified rPrP was calculated using OD₂₈₀ reading and the ϵ_{280} molar extinction coefficients of 63,370 for mouse PrP²³⁻²³⁰, according to the ExPASy Protein Server (<http://us.expasy.org/tools/protparam.html>). Generally, 15–18 mg of rPrP could be recovered from one liter bacterial culture.

2.2. Preparation of POPG lipid vesicles by sonication

Two hundred fifty microliters of 10 mg/ml 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-sn-glycerol) (sodium salt) (POPG) (Avanti Polar Lipids, Inc.) in chloroform was transferred into a Kimax glass tube (Fisher Scientific, Inc.) using a glass pipet. POPG in the tube was dried under a stream of nitrogen at 42°C and then hydrated in 1 ml of 20 mM Tris-HCl buffer (pH 7.4) to reach a final concentration of 2.5 mg/ml. Hydrated POPG lipid solution was vortexed and sonicated in a cup-hold sonicator (model XL2020, Misonix Inc.) until clear. POPG lipid vesicles were kept under argon in the glass tube with a rubber-lined screw cap at 4°C .

2.3. Isolation of total RNA from mouse liver

One hundred milligram normal mouse liver in 1 ml RNAzol (Tel-Test, Inc., Friendswood, Texas) was chopped by a razor blade and then thoroughly homogenized in a tissue grinder (Kontes) on ice. After adding 200 μl chloroform to the homogenate, the whole mixture was hand shaken for 30 seconds and kept at room temperature for 5 minutes. The mixture was then centrifuged at $12,000 \times g$ for 15 minutes at 4°C and the upper phase ($\sim 600 \mu\text{l}$) was transferred to a fresh tube containing 500 μl isopropyl alcohol. After vortex mixing, the mixture was incubated at room temperature for 5 minutes followed by a centrifugation at $12,000 \times g$ for 10 minutes at 4°C . After removing the supernatant, 1 ml 70% ethanol was added to the pellet and the tube was vortexed until the pellet was detached from the bottom. Then the sample was centrifuged at $7,000 \times g$ for 5 minutes at 4°C and the supernatant was discarded. The pellet was air dried for about 20 minutes at room temperature and resuspended in 100 μl Nuclease Free H₂O (Qiagen, USA). The OD₂₆₀/OD₂₈₀ ratio of isolated RNA was around 1.6 to 1.7 and the concentrations of RNA were calculated according to OD₂₆₀ readings (Nanodrop ND-1000, Thermo Scientific).

2.4. In vitro generation of rPrP-res

2.4.1. Protein Misfolding Cyclic Amplification (PMCA)—Protein Misfolding Cyclic Amplification (PMCA) was first designed to amplify the misfolded PrP^{Sc}, using PrP^{Sc} molecules in diseased brain homogenate as templates and PrP^C molecules in normal brain homogenate as substrates (8). In seeded PMCA reactions, both the PK-resistant PrP^{Sc} form and prion infectivity were amplified *in vitro*, mimicking the propagation of PrP^{Sc} *in vivo* (9). Recently, *de novo* formation of infectious prion, using either purified PrP^C or rPrP in the presence of facilitating factors, have been achieved using the PMCA technique (13,15).

2.4.2. PMCA instrument setup—A sonicator with a microplate horn (model S3000MP, Misonix, Inc.) was set up for PMCA reactions. The sonicator was connected to an Isotemp Refrigerated Circulator (Model 910, Fisher Scientific). The temperature of water in the circulator was set at 39°C so that the water temperature in the cup of microplate horn was around 37°C . All PMCA reactions were carried out in 8-strip thin-wall 200 μl PCR tubes (GeneMate, ISC BioExpress). The tubes were placed in a rack on top of the microplate horn with the bottom of PCR tubes about 3 mm above the horn surface. The level of circulating

water in the cup was adjusted to cover the reaction mixture in the PCR tubes. During the reaction, the cup was covered by a sheet of plastic wrap to prevent water evaporation. The sonicator was programmed to perform 48 repeated cycles of 30 second sonication followed by 29.5 minute incubation and the output was set at 6.0. One round PMCA was a 24-hour reaction consisting of 48 cycles of sonication and incubation.

2.4.3. Preparation of PMCA substrate—Purified rPrP was thawed and centrifuged ($100,000 \times g$, 1 hour, 4°C). The supernatant was diluted with deionized water to 0.25 mg/ml. In a 1.5 ml siliconized eppendorf tube (Midsco, St. Louis), 180 μl of diluted rPrP was mixed with 16 μl of POPG (2.5 mg/ml in 20 mM Tris-HCl, pH 7.4) and incubated at room temperature for 10 minutes. During the incubation, 500 μl of 5% Triton X-100 and 900 μl of $10 \times \text{TN}$ buffer (100 mM Tris-HCl, pH 7.5, 1.5M NaCl) was added to 7359 μl of Nuclease Free H_2O (Qiagen, USA) in a 15 ml centrifuge tube (GeneMate, ISC BioExpress) to prepare the substrate buffer. The rPrP-POPG mixture was transferred to the 15 ml centrifuge tube containing substrate buffer. The mixture was mixed thoroughly and incubated at room temperature for 5 minutes. At last, 45 μl of mouse liver RNA (6 mg/ml in Nuclease free H_2O) was added to the substrate mixture and thoroughly mixed (Table 1). The prepared PMCA substrate was aliquoted into the PCR tubes (90 μl per tube) and stored at -80°C .

2.4.4. De novo rPrP-res formation—The PMCA substrates was thawed at room temperature and briefly centrifuged. For first-round reaction, 10 μl of 1% Triton X-100 in PBS (without Mg^{++} and Ca^{++}) was added to the PMCA substrate. After the first round, the reaction tubes were briefly centrifuged to ensure that all the liquid was at bottom of the tube. PMCA product was mixed by pipetting up and down 20 times to ensure homogeneity. After mixing, 10 μl of first round product was transferred to a tube containing fresh PMCA substrate for another round of PMCA reaction. The same procedure was repeated for serial PMCA reactions.

2.4.5. Propagation of rPrP-res—PMCA products containing rPrP-res were used as seed for PMCA reactions to propagate rPrP-res. The procedure and the reaction conditions were the same as that described for *de novo* rPrP-res formation.

2.4.6. PK digestion of PMCA products—After one round PMCA reaction (either *de novo* rPrP-res formation or rPrP-res propagation), tubes were briefly centrifuged. After mixing the PMCA product by pipeting up and down for 20 times, 30 μl of each sample was transferred to a clean 1.5 ml eppendorf tube and 10 μl PK (100 ug/ml in the PK storage buffer containing 50 mM Tris, 1 mM CaCl_2 , pH 8.0) was added into each tube. The tubes were vortexed, briefly centrifuged, and incubated in a 37°C water bath at for 30 minutes followed by 5 minute incubation on ice. To terminate PK digestion, 2 μl of 100 mM Phenylmethanesulfonyl fluoride (PMSF) was added into each tube and tubes were vortexed and incubated on ice for additional 5 minutes. For precipitating all the proteins, 4 μl Bovine Serum Albumin (BSA) (5 mg/ml in deionized water) was added into each tube as a carrier protein and then 200 μl of cold methanol (-20°C) was added into each tube. After mixing by inverting the tube several times, the tubes were kept at -20°C for at least 45 minutes. After a 15 minute centrifugation at $16,300 \times g$ at 4°C , the pellet was resuspended in 20 μl of $1 \times \text{SDS-PAGE}$ sample buffer (65 mM Tris-HCl, pH 6.8, 5% SDS, 3% β -mercaptoethanol, 10% glycerol and trace amount of Bromophenol Blue) and boiled at 100°C for 10 minutes. Samples were loaded onto a 14% SDS-PAGE gel and electrophoresed.

2.4.7. Detection of rPrP-res by immunoblot—After electrophoresis, the SDS-PAGE gel was soaked in transferring buffer (25 mM Tris-HCl, 192 mM glycine, 20% methanol) for 10 minutes. Meanwhile, PVDF membrane was soaked sequentially in pure methanol for

15 seconds, in deionized water for 5 minutes, and in transferring buffer for 10 minutes. The wet transferring apparatus (Bio-Rad Laboratories) was assembled according to the manufacturer's direction and the transfer was carried out overnight with a constant voltage of 35 volts.

After transfer, the PVDF membrane was blocked with 5% nonfat milk in 0.1% Tween-20 in Tris-buffered Saline buffer (TBST) (50 mM Tris-HCl, 150 mM NaCl, pH 8.0, 0.1% Tween-20) for 1 hour at room temperature. The membrane was incubated at room temperature with primary antibody (1:2500 dilution of monoclonal POM1 anti-PrP antibody (28) in 2% nonfat milk in TBST) for 1 hour, washed three times with 2% nonfat milk in TBST (5 minutes each), incubated with secondary antibody (1:5000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Bio-Rad Laboratories) in 2% nonfat milk in TBST) for 1 hour, and washed three times with TBST (10 minutes each). The immunoblot was developed with ECL-plus reagent (GE Healthcare Life Science) according to manufacturer's direction and the signal was detected by exposure to X-ray films (MidSci, Inc.) (Fig. 2)

Based on the quantification of the PK-resistant band on X-ray films, we estimated that around 10% of rPrP in PMCA substrate was converted into the rPrP-res conformation after one round of PMCA reaction seeded with rPrP-res.

2.5. Preparation of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Gel casting system (miniVE Vertical Electrophoresis Systems, GE Healthcare Life Science) was assembled according to the manufacture's operation guide. To prepare 14% separating gel, 3.5 ml of 40% Acrylamide/Bis (29:1), 2.5 ml of 1.5 M Tris-HCl (pH 8.8), 100 µl of 10% SDS, 50 µl of 10% ammonium persulfate (APS), 5 µl of N,N,N',N' tetramethylethylene diamine (TEMED), and 3.85 ml of deionized water were mixed together and loaded into the gel casting slot. Seventy-five percent ethanol was overlaid on top of the separating gel to prevent evaporation. The gel was polymerized at room temperature (usually ~30 minutes). To prepare 4% stacking gel, 250 µl of 40% Acrylamide/Bis (29:1), 630 µl of 0.5 M Tris-HCl (pH 6.8), 25 µl of 10% SDS, 12.5 µl of 10% APS, 2.5 µl of TEMED, and 1.59 ml of deionized water were mixed together. The ethanol on top of the separating gel was removed and the stacking gel was overlaid on top of the separating gel. A comb was inserted into the stacking gel and removed after the stacking gel polymerized. The gel running module was assembled into the buffer tank containing 1 liter of running buffer (3.0 g Tris, 14.4 g Glycine and 1 g SDS in 1 liter of deionized water) and running buffer was added to the upper buffer chamber of the gel running module. After loading the samples, the electrophoresis was carried out at a constant voltage of 150 volts and stopped when the loading dye is about 1 cm above the lower end of the gel.

2.6. Silver staining of SDS-PAGE gel

Silver staining procedures were performed on an orbital shaker with proper shaking at room temperature.

Buffers used in silver staining are listed below.

Fixer solution: 250 ml of methanol, 60 ml of acetic acid (Glacier), 250 µl of formaldehyde (37.5% solution) and deionized water were mixed together to make a 500 ml of fixer solution.

Pretreatment solution: 1.5 ml of 1 M Na₂S₂O₃ was mixed together with 298.5ml deionized water to make 300 ml of pretreatment solution.

Silver solution: 0.4 g of AgNO_3 and 100 μl of formaldehyde (37.5% solution) were added into deionized water to make 200 ml of silver solution.

Reducing solution: 9.54 g of Na_2CO_3 , 45 μl of 1 M $\text{Na}_2\text{S}_2\text{O}_3$, and 150 μl of formaldehyde (37.5% solution) were mixed together with deionized water to make 300 ml of reducing solution.

The SDS-PAGE gel was sequentially soaked in 150 ml of fixer solution for 30 minutes, twice in 150 ml of 50% Ethanol (15 minutes each) and in 150 ml of pretreatment solution for 1 minute. Then the gel was rinsed with 150 ml deionized H_2O for three times (20 seconds each), incubated in 200 ml silver solution for 20 minutes and rinsed in 150 ml deionized H_2O twice (20 seconds each). Afterwards, the gel was incubated in 150 ml reducing buffer for 10 seconds and then incubated in a fresh 150 ml reducing buffer until the signal reached the desired intensity. The staining was stopped by rinsing the gel with deionized H_2O .

2.7. Intracerebral inoculation of wild type mice with rPrP-res

2.7.1. Preparation of inoculum—*For rPrP-res inoculation*, a 5-ml pool of rPrP-res seeded PMCA reaction product was laid on top of a 500 μl 10% (w/v in PBS) sucrose cushion and centrifuged at $199,000 \times g$ for 1 hour at 4°C . After removing the supernatant, the pellet was resuspended in 5 ml sterilized PBS, laid over another 500 μl 10% sucrose cushion and centrifuged at $199,000 \times g$ for 1 hour at 4°C . The pellet-washing step was repeated one more time. Pellet after second wash was resuspended in 625 μl sterilized PBS containing 1% BSA, and sonicated in a water-cooled cup-horn sonicator (model XL2020, Misonix, Inc.) for 5 minutes before injection.

2.7.2. Intracerebral injection—Each 8-week-old female CD-1 mouse (Harlan) was anesthetized and inoculated intracerebrally with 30 μl inoculum. The whole procedure was performed within a biosafety cabinet and the injection was carried out using a 1-ml disposable syringe with a 25-gauge disposable needle. The injection site was in the right parietal lobe, which was 1 mm right to the sagittal suture and 1 mm anterior to the lambdoid suture.

2.8. Histopathological analysis of aggregated PrP in diseased mouse brains

2.8.1. Tissue fixing, decontamination and processing—Sagittally dissected half brains were fixed in 4% formaldehyde in PBS at 4°C for 2 days, and decontaminated by submerging in 88% formic acid for 1–1.5 hours. Afterwards, the brains were fixed by 4% formaldehyde in PBS at 4°C for additional 1–5 days. The brains then were washed twice with PBS at room temperature (30 minutes each) and stepwise dehydrated by 50%, 75%, 85%, 95% (30 minutes each), and 100% ethanol (30 minutes, twice). The brains were cleared by submerging in xylene twice at room temperature (15 minutes each), followed by Xylene/paraffin (1:1) at 60°C for 45 minutes. Finally, the brains were immersed in first paraffin tank at 60°C for 1 hour and then switched to the second paraffin tank for another 3 to 4 hours at 60°C before paraffin embedding.

2.8.2. Immunohistochemical staining of aggregated PrP in diseased brains—Five- μm -thick sections were deparaffinized with treatments in xylenes for 3 times (5 minutes each), rehydrated with 100, 95, 75, 50% ethanol and dd H_2O (3–5 minute each). Antigen retrieve was performed by incubating sections in 88% formic acid for 5 minutes at room temperature, washing in dd H_2O twice (5 minutes each), and autoclaving at 121°C for 12 minutes in a Ritter Speedclave (Ritter, Rochester NY). Slides were in a coplin jar containing dd H_2O to cover all the sections during autoclaving. Afterwards, the coplin jar with autoclaved slides was placed on the bench to cool down until the temperature was

lower than 42°C (~30 minutes). Cooled sections were equilibrated in PBS twice (5 minutes each) and treated with 3% H₂O₂ in PBS for 10 minutes to block the endogenous peroxidase activity. After two washes in PBS (5 minutes each), the nonspecific binding sites were blocked with 5% normal goat serum in PBS at room temperature for 20 minutes. Afterwards, sections were incubated with primary antibody (1:500 dilution of SAF84 monoclonal anti-PrP antibody (Cayman Chemical) in Amplifying Antibody Dilution Buffer (ProHisto)) by slow shaking at room temperature for 1 hour followed by overnight at 4°C. Next morning, the sections were placed on bench to let the temperature warm up to room temperature. Afterwards, the sections were washed with Amplifying IHC Wash Buffer (ProHisto) three times (5 minutes each). The incubation with secondary antibody (1:200 dilution of anti-mouse secondary antibody (Vector Lab) in Amplifying Antibody Dilution Buffer (ProHisto)) was carried out at room temperature for 45 minutes by slow shaking. After secondary antibody incubation, sections were washed with Amplifying IHC Wash Buffer (ProHisto) for 3 times (5 minutes each), treated with ABC reagents (Vector Lab) at room temperature for 45 minutes by slow shaking, and then washed again with Amplifying IHC Wash Buffer for 3 times (5 minutes each). Color was developed by incubating the sections with freshly prepared DAB solution (Vector Lab, prepared according to the manufacturer's direction) at room temperature for around 5 minutes, and the reaction was terminated by submerging the sections in ddH₂O. Sections were counterstained by Harris Hematoxylin (Sigma) for 1 minute and tap water rinsed for about 3 minutes. Sections were dehydrated by submerging in 75, 95 and 100% ethanol, cleared by submerging in xylenes and mounted with Permount (Fisher). Images were taken by an Olympus IX81 microscope (Fig. 3).

3. Concluding remarks

The development of the PMCA technique is a landmark advance in the prion field (9,13,15). With the PMCA technique, the recombinant mouse PrP was successfully converted into an aggregated and PK-resistant rPrP-res conformation in the presence of anionic lipid and RNA molecules. Moreover, the rPrP-res was capable of propagating through conversion of either rPrP *in vitro* or PrP^C *in vivo*, and inducing *bona fide* prion disease in wild type mice (15). These results provide a strong support for the Prion hypothesis (1).

Convenient accessibility of recombinant prion in large scale removes the first obstacle in determining the molecular structure of the infectious prion, which may reveal the molecular basis for the puzzling prion infectivity. Propagation of recombinant prion *in vitro* is also a convenient assay for elucidating the molecular mechanism of PrP conversion. Moreover, this assay will allow us to explore the mechanisms behind the intriguing species barrier and strain phenomena of prion infectivity. We also foresee the adaptation of this assay to a high throughput format, which will greatly enhance our ability to search for effective diagnostic or therapeutic agents against these fatal neurodegenerative diseases.

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Abbreviations

PrP	prion protein
rPrP	recombinant mouse PrP

rPrP-res	the proteinase K-resistant recombinant PrP form that is associated with prion infectivity
PMCA	protein misfolding cyclic amplification

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Figure 1.
Silver staining of purified rPrP on a 14% SDS-PAGE gel.

Figure 2.

De novo generation of rPrP-res by PMCA. At round 1, 10 μ l of 1% Triton X-100 in PBS (without Mg^{++} and Ca^{++}) was added to PMCA substrate, and 10 μ l PMCA product of round 1 was used to seed round 2 PMCA substrate, and so forth. PMCA products of all rounds were subject to 25 μ g/ml PK digestion. The rPrP-res band was detected by immunoblot analysis using the POM1 anti-PrP antibody.

Figure 3.

Immunohistochemical staining of aggregated PrP in diseased mouse brain. Mice received intracerebral rPrP-res injection developed classic manifestations of prion disease and sacrificed at terminal stage. Aggregated PrP was detected in various brain regions and this brain section revealed aggregated PrP in the thalamus, which was stained by SAF84 anti-PrP antibody (brown staining) and counterstained by Hematoxylin. Spongiosis, as shown in this brain section, was detected in multiple brain regions.

Table 1

PMCA substrate

rPrP (0.25 mg/ml, in deionized water)	180 µl
POPG (2.5 mg/ml, in 20 mM Tris, pH 7.4)	16 µl
10 × TN buffer (1.5 M NaCl, 100 mM Tris, pH 7.5)	900 µl
5% Triton X-100	500 µl
Nuclease free water	7359 µl
Mouse liver RNA (6 mg/ml)	45 µl
PMCA substrate	9000 µl