

De novo generation of infectious prions with bacterially expressed recombinant prion protein

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ABSTRACT The prion hypothesis is strongly supported by the fact that prion infectivity and the pathogenic conformer of prion protein (PrP) are simultaneously propagated *in vitro* by the serial protein misfolding cyclic amplification (sPMCA). However, due to sPMCA's enormous amplification power, whether an infectious prion can be formed *de novo* with bacterially expressed recombinant PrP (rPrP) remains to be satisfactorily resolved. To address this question, we performed unseeded sPMCA with rPrP in a laboratory that has never been exposed to any native prions. Two types of proteinase K (PK)-resistant and self-perpetuating recombinant PrP conformers (rPrP-res) with PK-resistant cores of 17 or 14 kDa were generated. A bioassay revealed that rPrP-res^{17kDa} was highly infectious, causing prion disease in wild-type mice with an average survival time of about 172 d. In contrast, rPrP-res^{14kDa} completely failed to induce any disease. Our findings reveal that sPMCA is sufficient to initiate various self-perpetuating PK-resistant rPrP conformers, but not all of them possess *in vivo* infectivity. Moreover, generating an infectious prion in a prion-free environment establishes that an infectious prion can be formed *de novo* with bacterially expressed rPrP.—Zhang, Z., Zhang, Y., Wang, F., Wang, X., Xu, Y., Yang, H., Yu, G., Yuan, C., Ma, J. *De novo* generation of infectious prions with bacterially expressed recombinant prion protein. *FASEB J.* 27, 4768–4775 (2013). www.fasebj.org

Key Words: sPMCA • recombinant PrP • bioassay

TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES (TSEs), also known as prion diseases, are a group of fatal

neurodegenerative disorders that can be manifested as sporadic, inherited, and infectious disorders. The prion hypothesis postulates that the infectious agent in TSEs is a prion, the scrapie form of prion protein (PrP^{Sc}), a pathogenic conformational isoform of the normal host cellular prion protein (PrP^C) (1). Because of its self-perpetuating property, PrP^{Sc} seeds the conversion of host PrP^C, resulting in a replication of the pathogenic PrP^{Sc} conformer and the subsequent pathological changes.

The normal PrP^C is a cell surface glycoprotein that is mainly α -helical, proteinase K (PK) sensitive, and soluble in mild detergents. In contrast, the disease-associated PrP^{Sc} is rich in β -sheet, PK resistant, and highly aggregated (1, 2). The development of the serial protein misfolding cyclic amplification (sPMCA) procedure, a technique involving successive cycles of alternating sonication and incubation (3, 4), has greatly advanced our understanding of the infectious agent. In particular it has been shown that the self-perpetuating PK-resistant prion protein (PrP) conformer and the prion infectivity can be propagated simultaneously by sPMCA with PrP^C in the crude brain homogenates (4), PrP^C purified from brains or cultured cells (5, 6), baculovirus-derived recombinant PrP (7), or purified bacterially expressed recombinant PrP (rPrP) (8–11), providing unequivocal experimental evidence supporting the prion hypothesis.

We previously showed that a highly infectious prion can be generated by sPMCA with a substrate mixture consisting of bacterially expressed murine rPrP, the synthetic phospholipid 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG), and total RNA isolated from normal mouse liver (8). Similar to disease-associated PrP^{Sc}, the sPMCA-generated PK-resistant rPrP (rPrP-res) form contains a C-terminal PK-resistant core, can be propa-

Abbreviations: BSL-2, biosafety level 2; dpi, days postinoculation; i.c., intracerebral; IPTG, isopropyl β -D-1-thiogalactopyranoside; PK, proteinase K; PMSF, phenylmethanesulfonyl fluoride; POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol; PrP, prion protein; PrP^C, cellular prion protein; PrP^{Sc}, scrapie form of prion protein; rPrP, recombinant prion protein; rPrP-res, proteinase K-resistant recombinant prion protein; PVDF, polyvinylidene fluoride; sPMCA, serial protein misfolding cyclic amplification; TSE, transmissible spongiform encephalopathy

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gated indefinitely by sPMCA *in vitro*, has the capability of infecting susceptible cultured cells, and causes prion disease in wild-type mice. Furthermore, our recent studies showed that infectious prions can be generated by replacing total RNA used in original study (8) with synthetic polyriboadenylic acid (9), or by using synthetic phosphatidylethanolamine as the solitary cofactor (10, 11). These results unambiguously revealed that a highly infectious prion can be generated with bacterially expressed rPrP plus defined synthetic cofactors. However, whether the rPrP-res can be initiated *de novo* by pure *in vitro* manipulations remains controversial. This uncertainty is largely due to the facts that the ultrasensitive sPMCA is capable of amplifying minuscule amounts of PrP^{Sc} (12–14), and there was limited usage of native prion (diseased mouse brain tissue) in the laboratory that generated the original rPrP-res (8). Because of the significance of *de novo* prion formation and the availability of a brand new laboratory at East China Normal University in Shanghai, we performed the *de novo* recombinant prion formation experiment in this new laboratory that has never been exposed to any native prions, eliminating the possibility of potential contamination.

MATERIALS AND METHODS

Reagents

Reagents used in this study included RNA STAT-60 (Tel-Test, Friendswood, TX, USA), Ni-NTA Superflow resin (Qiagen, Hamburg, Germany), polyvinylidene fluoride (PVDF) membrane and ECL reagents (Millipore, Billerica, MA, USA), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-sn-glycerol) (sodium salt; POPG, 16:0–18:1 PG in chloroform; Avanti Polar Lipids, Alabaster, AL, USA), PK (lyophilizate, recombinant, PCR grade; Roche, Indianapolis, IN, USA), and phenylmethanesulfonyl fluoride (PMSF) and isopropyl β -D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich, St Louis, MO, USA). Other chemicals were purchased from Sango Biotech Co. (Shanghai, China). Antibodies used in this study included 8B4 and M20 anti-PrP antibodies (Santa Cruz Biotechnology, Dallas, TX, USA), SAF32 anti-PrP antibody (Cayman Chemical, Ann Arbor, MI, USA), 8H4 anti-PrP antibody (a generous gift from Dr. Man-Sun Sy, Case Western Reserve University, Cleveland, OH, USA), HRP-conjugated goat anti-mouse IgG antibody (Bio-Rad, Hercules, CA, USA), HRP-conjugated horse anti-goat IgG antibody (Beijing Dingguo Changsheng Biotechnology, Beijing, China), and anti-GFAP antibody (Cell Signaling Technology, Danvers, MA, USA; or Dako, Carpinteria, CA, USA). The rPrP-expressing plasmid pET-22b moPrP23-230 was a generous gift from Dr. Surachai Supattapone (Dartmouth Medical School, Hanover, NH, USA).

Purification of rPrP

The pET22b moPrP23-230-transformed *Escherichia coli* BL21 (DE3) cells were cultured in 1 L Luria broth medium till OD₆₀₀ reached 0.5–0.6. Afterward, IPTG was added to reach a final concentration of 1 mM, and the culture was continued for 5 h. Induced *E. coli* cells were harvested (5000 g, 20 min, 4°C) and stored at –80°C.

For purification, *E. coli* cells were resuspended in 75 ml of buffer A (10 mM Tris–HCl and 100 mM NaPO₄, pH 8.0) and

lysed through 4 rounds of 3 min sonication on ice (amplitude=80, 2-s pulse on/1-s pulse off, 10 min incubation on ice between rounds; Misonix sonicator 3000; Misonix Inc., Farmingdale, NY, USA). Inclusion bodies were collected by centrifugation (10,000 g, 30 min, 4°C), resuspended in 75 ml of buffer B (6 M GuHCl, 10 mM Tris–HCl, 100 mM NaPO₄, and 10 mM BME, pH 8.0), and sonicated on ice till completely solubilized (4 to 6 rounds of 3 min sonication, amplitude=80, 2-s pulse on/1-s pulse off, 10 min incubation on ice between rounds). After centrifugation (10,000 g, 30 min, 4°C), the supernatant was mixed with 25 ml of Ni-NTA Superflow resin (preequilibrated with buffer B) and stirred for 1 h at room temperature. The resin was then packed into a glass chromatography column and washed with 120 ml of buffer B. Afterward, a 200 ml gradient of buffer B to buffer A was applied to the column (1.5 ml/min), which was followed by a wash with 75 ml buffer C (10 mM Tris–HCl, 100 mM NaPO₄, and 50 mM imidazole, pH 8.0). The rPrP was eluted with buffer D (10 mM Tris–HCl, 100 mM NaPO₄, and 500 mM imidazole, pH 5.8). The eluted fractions with OD₂₈₀ $\geq$ 0.1 were combined, added to dialysis tubing (8000 MWCO) and dialyzed for 1.5 h against buffer E (10 mM NaPO₄, pH 5.8, changing buffer every 30 min) and another 1.5 h against ddH₂O (changing ddH₂O every 30 min). Dialyzed rPrP in ddH₂O was kept at –80°C.

Preparation of POPG lipid vesicles and isolation of total RNA

Preparation of POPG vesicle and isolation of total RNA from FVB/NJ mouse liver were performed as described previously (8, 15).

Preparation of sPMCA substrate

After centrifugation at 100,000 g for 1 h at 4°C, the soluble rPrP concentration was determined using the DC protein assay kit (Bio-Rad). Here 864 μ l of soluble rPrP (0.46 mg/ml in ddH₂O) was mixed with 216 μ l of POPG (1 mg/ml in 20 mM Tris–HCl, pH 7.4) in a 1.5-ml RNase-free microcentrifuge tube and incubated at room temperature for 10 min. During the incubation, a mixture of 6420 μ l ddH₂O, 480 μ l of 5% Triton X-100, 960 μ l of 10 $\times$ TN buffer (1.5M NaCl and 100 mM Tris–HCl, pH 7.5), and 19.2 μ l of 500 mM EDTA was prepared in a 15-ml centrifuge tube. The rPrP-POPG mixture was transferred to the 15-ml centrifuge tube, thoroughly mixed, and incubated at room temperature for 5 min. After the incubation, 640 μ l of mouse liver RNA (2.4 mg/ml in RNase-free H₂O) was added. The substrate mixture was thoroughly mixed, portioned into aliquots in RNase-free PCR tubes (90 μ l/tube), and stored at –80°C. The concentrations of each component in the sPMCA substrate mixture were determined based on our previous studies of PrP-lipid interaction (16, 17), rPrP-res generation, and propagation (8, 9).

sPMCA instrument setup

A Misonix sonicator S-4000 with a microplate horn was used for sPMCA. All reactions were carried out in 8-strip thin-wall 200- μ l PCR tubes that were placed in a homemade rack in the microplate horn. The bottom of tubes were $\sim$ 3 mm above the horn surface. The sonicator was connected to a heated Cole-Parmer Polystat circulating water bath (Cole-Parmer, Vernon Hills, IL, USA), and the level of circulating water in the horn was adjusted to cover the reaction mixture in the PCR tubes. Water temperature is set at 39°C in the circulator so that the temperature of water bath in the microplate horn is at 37°C. During the reaction, the microplate horn was

TABLE 1. De novo rPrP-res formation by sPMCA

Experiment	Total samples	Total rounds of PMCA	Samples with <i>de novo</i> rPrP-res formation	Molecular mass of PK-resistant fragment (kDa)
1	8	24	0	—
2	8	24	0	—
3	8	24	8	14
4	4	12	4	17
5	8	20	6	17
6	4	20	0	—

covered with a plastic wrap to prevent evaporation. Sonicator was programmed to sonicate for 30 s followed by a 29.5-min incubation, and the amplitude of the sonicator was set at 65. For each round of PMCA, the sonication-incubation cycle repeated 48 times (24 h).

sPMCA

For the first round, 10 μ l PBS was added to the substrate. At the end of first round, the reaction product was mixed by pipetting, and 10 μ l of reaction product was transferred to a new tube containing 90 μ l substrate for the second PMCA round. The same transferring steps were repeated to carry out serial PMCA reactions.

Detection of rPrP-res in sPMCA products

The detection of rPrP-res was performed as described previously (8, 9, 15).

Mouse bioassay

Wild-type CD-1 mice were purchased from the Shanghai Laboratory Animal Center (Shanghai, China), and 6-wk-old male mice were used for all bioassays. Preparation of inocula, intracerebral (i.c.) inoculation, second-round transmission, and biochemical and pathological analyses of inoculated mice were performed as described previously (8, 9, 15). The mouse bioassay was performed at the specific pathogen-free animal facility at East China Normal University, and the i.c. inoculation was performed after mice were completely anesthetized. For all inoculations (including both rPrP-res^{14kDa} and rPrP-res^{17kDa}), the volume of inoculum was always 30 μ l/mouse, which equals 300 μ l of sPMCA products and contains 12.42 ng of total PrP. All mice were monitored daily, and efforts were made to minimize pain and suffering. All mouse experiments were performed according to the Guidelines on the Humane Treatment of Lab Animals established in 2006 by the Ministry of Science and Technology of China [policy (2006)398] and approved by the Institutional Animal Care and Use Committee of East China Normal University (approval protocol AR2009/10005). Histopathological analyses were performed as described previously (8, 18).

Biosafety

All experiments were performed at designated biosafety level 2 (BSL-2) laboratories at East China Normal University or at Ohio State University, following the U.S. Centers for Disease Control and Prevention guidelines for experiments involving mouse prions (19). All sPMCA products were kept at designated -80°C freezers in BSL-2 labs clearly marked with biohazard signs. All personnel were trained to work in a BSL-2 laboratory.

RESULTS

De novo formation of rPrP-res

The unseeded sPMCA was performed with a substrate mixture consisting of murine rPrP, POPG, and total RNA isolated from normal mouse liver, and the *de novo* formation of rPrP-res was detected by immunoblot analyses of PK-digested sPMCA products. In a total of 6 tries (4–8 samples and 12–24 rounds of sPMCA per experiment; Table 1), the successful generation of rPrP-res was detected in 3 separated unseeded sPMCA experiments (Fig. 1 and Table 1). The initiation of the rPrP-res conformer appeared to be random, and we detected the first appearance of rPrP-res at different sPMCA rounds, ranging from round 5 to round 18 (Fig. 1, Supplemental Fig. S1, and data not shown). Interestingly, rPrP-res with 2 different sizes of PK-resistant core,

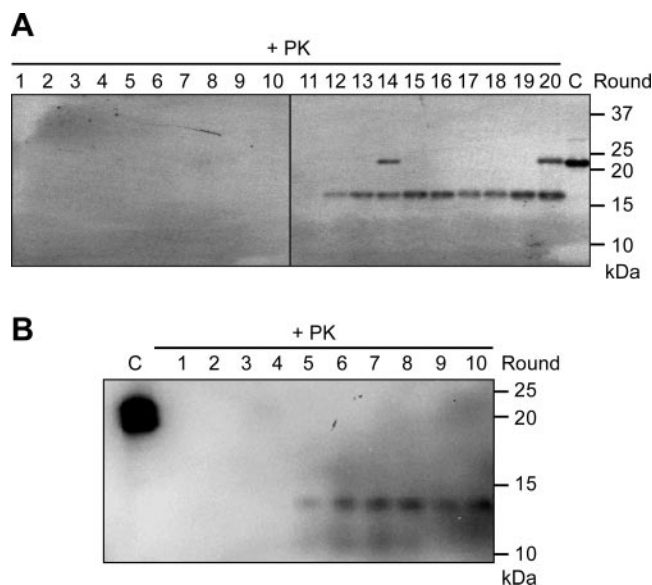


Figure 1. *De novo* formation of rPrP-res. The sPMCA reaction was carried out with a substrate consisting of rPrP, POPG, and total RNA isolated from normal mouse liver. The presence of rPrP-res was determined by subjecting the sPMCA product to 50 μ g/ml PK digestion at 37°C for 30 min, followed by immunoblot analysis. A) *De novo* formation of rPrP-res^{17kDa} detected by the 8H4 antibody. B) *De novo* formation of rPrP-res^{14kDa} detected by the M20 antibody. Numbers at top indicate sPMCA rounds. C, control (undigested sPMCA substrate). The full-length rPrP detected in lanes 14 and 20 likely resulted from incomplete PK digestion.

14 kDa (rPrP-res generated in experiment 3) and 17 kDa (rPrP-res generated in experiments 4 and 5), were detected (denoted as rPrP-res^{14kDa} and rPrP-res^{17kDa}). Once formed, both rPrP-res forms are able to propagate indefinitely by sPMCA.

Comparing the biochemical properties of rPrP-res^{17kDa} and rPrP-res^{14kDa}

Similar to naturally occurring PrP^{Sc}, both rPrP-res forms were aggregated (Fig. 2A, D) and PK resistant (Fig. 2B, E). The PK-resistant rPrP fragments were still detectable after a 200 µg/ml PK digestion at 37°C for 30 min, indicating a relatively high PK resistance. A panel of anti-PrP antibodies was used to probe the PK-resistant fragments of the 2 rPrP-res forms to determine whether they have a C-terminal PK resistance similar to naturally occurring PrP^{Sc} (2). For rPrP-res^{17kDa}, the PK-resistant core was not recognized by the 8B4 antibody (recognizing an N-terminal epitope at aa 35–45; refs. 20, 21), but was detected by SAF32, 8H4, and M20 antibodies (Fig. 2C). Since SAF32 antibody recognizes the octarepeat region (22) and the epitopes of 8H4 and M20 antibodies are localized at the C terminus of PrP (21, 22), we concluded that the PK-resistant core of rPrP-res^{17kDa} is similar to that of naturally occurring PrP^{Sc}, from the C terminus of the octarepeat region to the C-terminal end of PrP (23). Surprisingly, the PK-resistant core of rPrP-res^{14kDa} was detected only by M20 antibody, but not by any of the other 3 antibodies (Fig. 2F). Considering the size of the PK-resistant fragment (~14 kDa) and the location of

8H4 epitope (aa 175–185; refs. 20, 21), the inability of this antibody to recognize the PK-resistant core of rPrP-res^{14kDa} likely indicates that the rPrP-res^{14kDa} conformation is different from that of rPrP-res^{17kDa}, resulting in the inaccessibility of 8H4 epitope by immunoblot detection. The conformational-dependent exposure of the 8H4 epitope is consistent with previously reported renaturation of the conformational PrP epitope during the process of electrophoresis and/or immunoblot analysis (24). Nevertheless, the detection by M20 antibody (Fig. 2F) does support the hypothesis that the PK-resistant core of rPrP-res^{14kDa} is also at the C terminus of PrP.

The rPrP-res^{17kDa}, but not rPrP-res^{14kDa}, causes prion disease in wild-type mice

To determine whether the newly formed rPrP-res forms are capable of causing prion disease in animals, we intracerebrally inoculated wild-type CD-1 mice with rPrP-res^{14kDa} or rPrP-res^{17kDa}. The sPMCA substrate mixture without going through sPMCA or inoculation diluent (1 mg/ml BSA in PBS) was used as the control inoculum. Mice that received i.c. inoculation with rPrP-res^{17kDa} developed neurological signs of prion disease, including claspings, tail plasticity, hypokinesia, kyphosis, and mild ataxia and became listless and cachectic at the terminal stage. The survival times for rPrP-res^{17kDa}-inoculated mice ranged from 161 to 185 days postinoculation (dpi), and the average survival time was 172.3 ± 1.6 dpi (means ± SE; Fig. 3A and Table 2). None of the control mice that received i.c. inoculation with control

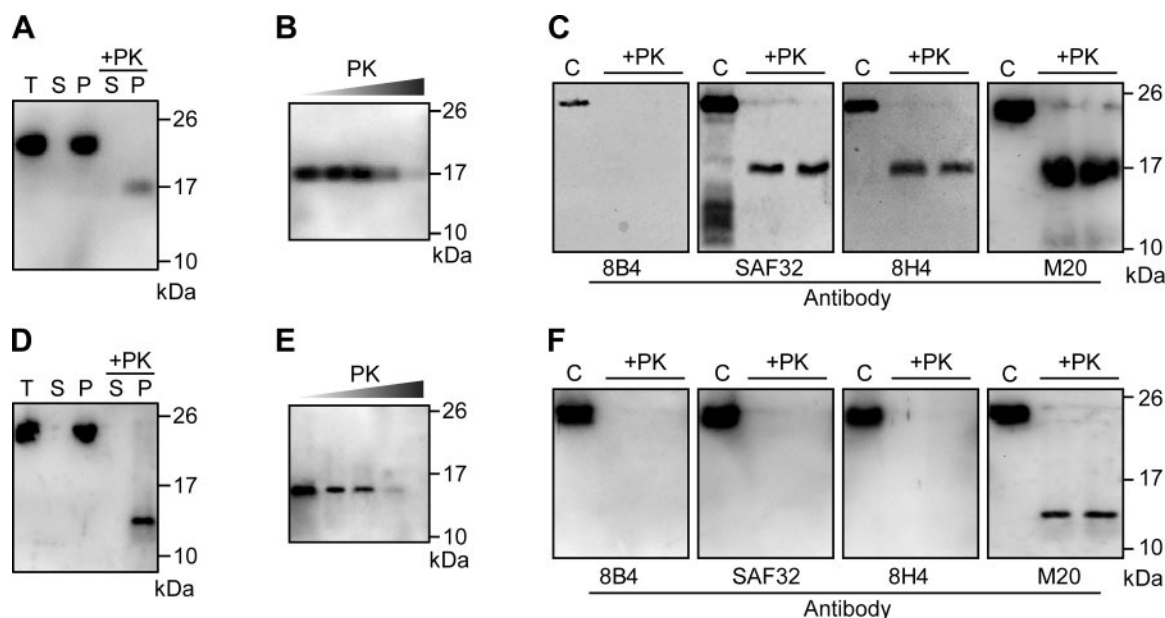


Figure 2. Biochemical characterization of rPrP-res^{17kDa} and rPrP-res^{14kDa}. A, D) rPrP-res^{17kDa} (A) and rPrP-res^{14kDa} (D) propagated by sPMCA were separated into supernatant (S) and pellet (P) fractions by centrifugation for 1 h at 100,000 g at 4°C. Amounts of rPrP in the supernatant and pellet fractions were determined by immunoblot analysis (lanes 2 and 3). Amounts of rPrP-res in the supernatant and pellet fractions were determined by PK digestion and immunoblot analysis (lanes 4 and 5). T, total rPrP input. B, E) Serial PK digestion of rPrP-res^{17kDa} (B) and rPrP-res^{14kDa} (E). Concentrations of PK were 25, 50, 100, 200, and 400 µg/ml; digestion was carried out at 37°C for 30 min. C, F) rPrP-res^{17kDa} (C) and rPrP-res^{14kDa} (F) were digested with PK and detected by immunoblot analysis with various antibodies as indicated. C, control (undigested sPMCA substrate). rPrP-res^{17kDa} (A, B) and rPrP-res^{14kDa} (D, E) were detected by 8H4 and M20 antibody, respectively.

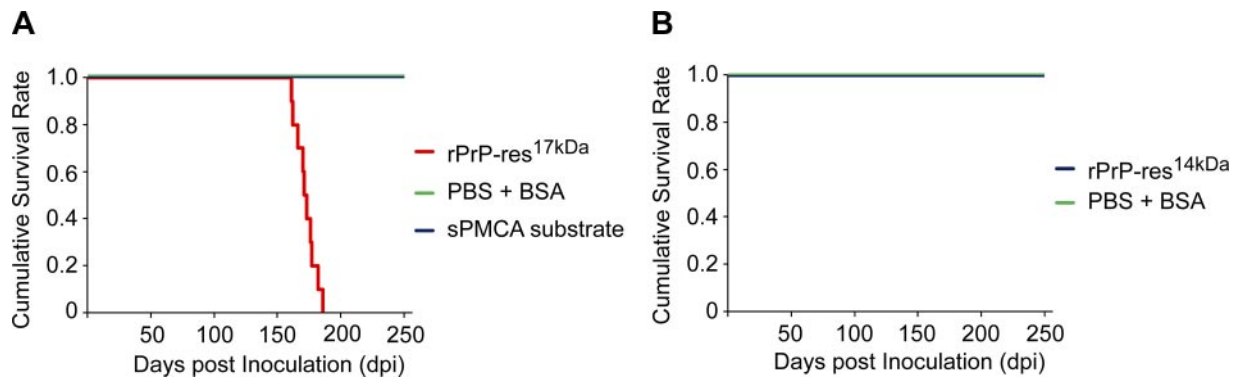


Figure 3. Survival plots. *A*) Survival plot of CD-1 mice inoculated with rPrP-res^{17kDa} and control inocula, sPMCA substrate, and inoculum diluent (PBS+BSA). *B*) Survival plot of CD-1 mice inoculated with rPrP-res^{14kDa} and inoculum diluent (PBS+BSA).

inocula developed disease (Fig. 3A). Although the survival time of rPrP-res^{17kDa}-inoculated mice appeared to be longer than the average survival time of 150 ± 2.2 dpi for rPrP-res^{OSU}, the rPrP-res generated in our previous study at Ohio State University (8), it was still within the variation among different prion inoculations (25). More interestingly, i.c. inoculation of rPrP-res^{14kDa} in wild-type CD-1 mice did not cause any signs of disease (Fig. 3B; the last rPrP-res^{14kDa}-inoculated mouse was euthanized at 602 dpi).

Analyses of mouse brain tissues confirmed the presence of spongiosis, astrogliosis, and PK-resistant PrP^{Sc} in the brains of rPrP-res^{17kDa}-inoculated mice (Fig. 4A, B), but not in rPrP-res^{14kDa}-inoculated mouse brains (Fig. 4C, D). Similarly, the aberrant PrP deposit was observed only in rPrP-res^{17kDa}-inoculated mice (data not shown). The glycosylated and glycosphosphatidylinositol (GPI)-anchored PrP^{Sc} in rPrP-res^{17kDa}-inoculated mouse brains migrated around 19–26 kDa and was readily detected by the 8H4 antibody (Fig. 4B). In contrast, no PrP^{Sc} was detected in rPrP-res^{14kDa}-inoculated mouse brains (Fig. 4D). Since the PK-resistant fragment of rPrP-res^{14kDa} was recognized only by M20 antibody (Fig. 2F), we repeated the immunoblot analysis of PK-digested brain homogenates with M20 antibody and confirmed that there was no PK-resistant PrP band in the brain homogenates of rPrP-res^{14kDa}-inoculated mice (Fig. 4E). These results indicate that the

rPrP-res^{14kDa} could not seed endogenous PrP^C conversion *in vivo*.

Second-round transmission was performed by intracerebrally inoculating wild-type CD-1 mice with 1% brain homogenates prepared from rPrP-res^{17kDa}-inoculated mice. As expected, all mice developed disease, with an average survival time of 161.3 ± 1.8 dpi (Table 2 and Fig. 5A). Spongiosis, astrogliosis, and PK-resistant PrP^{Sc} were detected in the brains of mice that received second-round transmission (Fig. 5B, C). None of the mice that received i.c. inoculation of brain homogenate prepared from first-round control mice (which received i.c. inoculation of sPMCA substrate) developed disease (Table 2 and Fig. 5). Collectively, we concluded that the *de novo* generated rPrP-res^{17kDa} is highly infectious, capable of causing *bona fide* prion disease in wild-type CD-1 mice. The rPrP-res^{14kDa}, however, is not infectious to wild-type mice.

DISCUSSION

Recent studies have demonstrated the association of prion infectivity with various *in vitro* generated rPrP conformers, strongly supporting the prion hypothesis (8–11, 26–29). We previously showed that a highly infectious recombinant prion can be generated with

TABLE 2. Summary of infectivity bioassay in wild-type CD-1 mice

Inoculum	Diseased/ inoculated mice	Survival (dpi)	Average survival (d)
rPrP-res ^{17kDa}	10/10	161, 162, 166, 170, 171, 173, 176, 177, 182, 185	172.3 ± 1.6
Control 1: sPMCA substrate	0/5	>300	NA
Control 2: PBS + BSA	0/5	>300	NA
2nd passage of rPrP-res ^{17kDa} : 1% brain homogenates from mice inoculated with i.c. rPrP-res ^{17kDa}	7/7	146, 158, 161, 160, 170, 170, 174	161.3 ± 1.8
2nd passage of control mice: 1% brain homogenates from mice inoculated with sPMCA substrate	0/5	>250	NA
rPrP-res ^{14kDa}	0/9	>365	NA
Control: PBS + BSA	0/5	>365	NA

Average survival values are means $\pm$ SE. NA, not applicable.

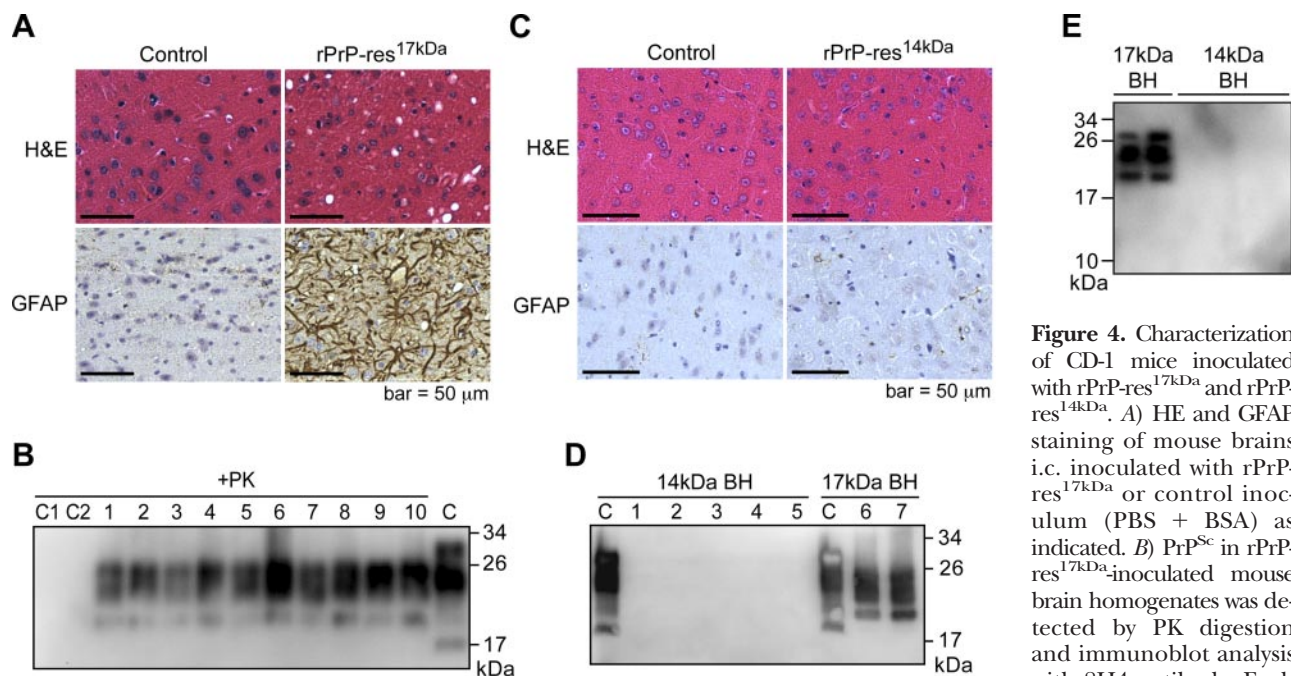


Figure 4. Characterization of CD-1 mice inoculated with rPrP-res^{17kDa} and rPrP-res^{14kDa}. **A)** HE and GFAP staining of mouse brains i.c. inoculated with rPrP-res^{17kDa} or control inoculum (PBS + BSA) as indicated. **B)** PrP^{Sc} in rPrP-res^{17kDa}-inoculated mouse brain homogenates was detected by PK digestion and immunoblot analysis with 8H4 antibody. Each

number represents an individual inoculated mouse. C = undigested mouse brain homogenate was used as a control. C1 and C2 are brain homogenates prepared from mice intracerebrally inoculated with control inocula, inoculum diluent (PBS+BSA) and sPMCA substrate, respectively. **C)** HE and GFAP staining of mouse brains intracerebrally inoculated with rPrP-res^{14kDa} or control inoculum (PBS+BSA) as indicated. **D)** Brain homogenates (BH) prepared from rPrP-res^{14kDa}-inoculated mice (14-kDa BH) and rPrP-res^{17kDa}-inoculated mice (17-kDa BH) were subjected to PK digestion and immunoblot analysis with 8H4 antibody. **E)** PK-resistant PrP^{Sc} in the brain homogenates prepared from mice inoculated with rPrP-res^{17kDa} (17-kDa BH) and rPrP-res^{14kDa} (14-kDa BH) was detected by immunoblot analysis with M20 antibody.

rPrP plus POPG and total mouse liver RNA *via* an unseeded sPMCA reaction (8). To determine whether the infectious rPrP-res can indeed be generated *de novo*, the current study was carried out in a new prion-free laboratory, eliminating the possibility of contamination. Our results clearly showed that an infectious recombinant prion can be generated *de novo*, which causes prion disease in wild-type mice with a 100% attack rate and a relatively synchronized survival time.

Compared to rPrP amyloid fiber prepared by various approaches (27–29), the sPMCA-generated recombi-

nant prions generally contain higher infectivity (8–11, 26), suggesting that the sPMCA procedure, cyclic sonication and incubation, contributes to the formation of an infectious prion. The sPMCA was originally developed based on the notion that sonication-fragmented PrP^{Sc} aggregates would increase the seeding efficiency (3), which readily explains the PrP^{Sc}-seeded prion propagation, but cannot account for the *de novo* prion formation. We propose that the *de novo* prion formation in our system is due to the ability of sPMCA to reassemble rPrP aggregates during each cycle of PMCA, *i.e.*,

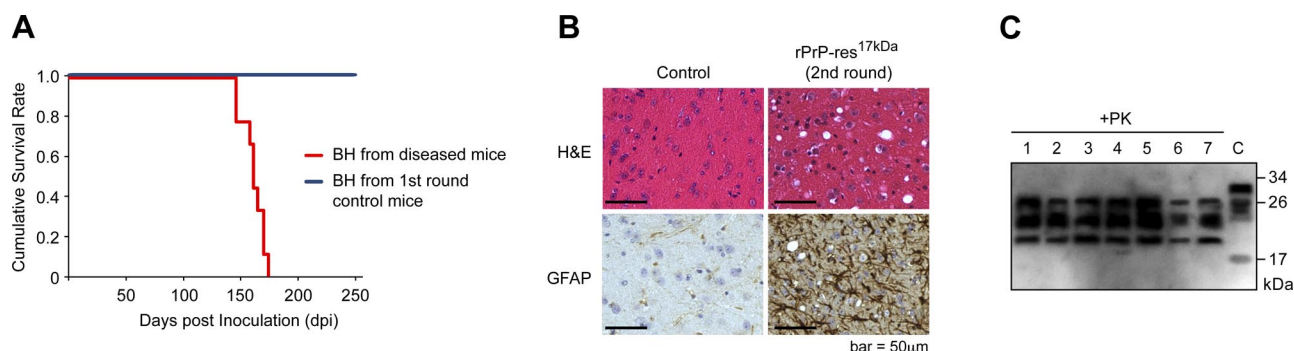


Figure 5. Second-round transmission of rPrP-res^{17kDa}. **A)** Survival plots of mice inoculated with 1% brain homogenates (BH) prepared from rPrP-res^{17kDa} inoculated mice or 1% brain homogenates (BH) prepared from control mice received inoculation of sPMCA substrate. **B)** Pathological analysis of mice received second-round transmission. The rPrP-res^{17kDa}-inoculated and control mouse brain sections were stained with hematoxylin and eosin (H&E) or an anti-GFAP antibody as indicated. **C)** PrP^{Sc} in brain homogenates prepared from mice that received second-round transmission of rPrP-res^{17kDa} was detected by PK digestion followed by immunoblot analysis with 8H4 antibody. C, control (undigested mouse brain homogenate).

breaking PrP aggregates by sonication and reforming PrP aggregates during the incubation. The sPMCA substrate used in our system contains 2 cofactors, POPG and RNA (8, 30), both of which are known to induce rPrP unfolding and aggregation (16, 17, 31–36). Particularly, we have shown that the rPrP-POPG interaction results in a β -sheeted rPrP conformer with PrP^{Sc}-like PK-resistance (16). Thus, the rPrP in our sPMCA substrate mixture is likely in an aggregated and β -sheeted conformational state similar to the infectious PrP^{Sc} conformer, but without the seeding capability. When this substrate is subjected to sPMCA, cycles of sonication and incubation will lead to the breakup and reassembly of rPrP aggregates. Because of the lack of seeding during the *de novo* prion formation process, the PrP reaggregation process is random, resulting in packaging of a variety of PrP aggregates during each PMCA cycle. However, if one of the newly packaged rPrP aggregates possesses the self-perpetuating property, it will act as a seed to guide the rPrP reaggregation and gradually become the dominantly propagated rPrP species, resulting in the formation of a self-perpetuating PrP conformer. This model explains the *de novo* recombinant prion formation by sPMCA and the randomness of rPrP-res appearance in sPMCA (Fig. 1 and Supplemental Fig. S1). It also accounts for the previous studies showing *de novo* prion formation with purified PrP^C (5) or brain homogenates (37), since both cofactors, lipids and RNA, are present in the sPMCA substrates used in those studies.

Although the lipid- and/or RNA-interacted rPrP in the sPMCA substrate is already in a conformation similar to the final infectious PrP conformation, further rPrP conformational changes during the assembly of self-perpetuating rPrP aggregates cannot be excluded. In fact, the formation of different types of rPrP-res with the same substrate mixture in our current and previous studies (8) supports the hypothesis that there are further rPrP conformation alterations during sPMCA-mediated assembly.

The rPrP-res^{14kDa} generated in this study is clearly self-perpetuating *in vitro*, but it failed to cause any disease in wild-type mice. The shorter PK-resistant core and the inaccessibility of the 8H4 epitope (Fig. 2F) suggest that the conformation of rPrP-res^{14kDa} is completely different from that of rPrP-res^{17kDa} or rPrP-res^{OSU}. Since the sPMCA substrate mixtures used for *de novo* formation of rPrP-res^{14kDa} and rPrP-res^{17kDa} were identical, the generation of totally different rPrP-res forms could be due to the randomness of sPMCA-mediated rPrP aggregation process, the particular sonication power output during that experiment (the power output varies despite always setting the amplitude of the sonicator at 65), or the combination of both. If the variation in sonication power output does play a role, it would indicate that different physical treatments can lead to different rPrP conformations (or differently packed rPrP aggregates), which would be consistent with the previous reports showing that different shaking modes generate distinct types of rPrP amyloid fibers (38) and that different sonication powers are required to propagate different prion strains (14).

Because of its ability to amplify minute amounts of the disease-associated PrP^{Sc} conformer, sPMCA is considered one of the most promising diagnostic approaches for prion disease and has been demonstrated to sensitively detect PrP^{Sc} in body fluids of rodent prion models (39–41). However, the ability of sPMCA to generate infectious prion *de novo* encourages caution in applying sPMCA for the diagnostic purpose.

Collectively, our results reveal that sPMCA is capable of forming various self-perpetuating rPrP-res conformers *de novo*, and some of these rPrP conformers, such as rPrP-res^{17kDa} and rPrP-res^{OSU}, are sufficient to cause prion disease in wild-type animals. In addition to understanding the origin of infectious mammalian prions, our findings may also have important implications in understanding the relationship between *in vitro* self-perpetuating protein conformers and the *in vivo* prion-like spread of pathology in various human diseases (42–48). **[F]**

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Supporting Information

Figure S1 *De novo* formation of rPrP-res^{17kDa} in various samples. The sPMCA rounds were indicated. The PK-resistant rPrP-res^{17kDa} was detected by immunoblot analysis with 8H4 antibody.

