Mammalian Prions Generated from Bacterially Expressed Prion Protein in the Absence of Any Mammalian Cofactors*§

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Transmissible spongiform encephalopathies (TSEs) are a group of neurodegenerative diseases that are associated with the conformational conversion of a normal prion protein, PrP^C, to a misfolded aggregated form, PrP^Sc. The protein-only hypothesis asserts that PrP^Sc itself represents the infectious TSE agent. Although this model is supported by rapidly growing experimental data, unequivocal proof has been elusive. The protein misfolding cyclic amplification reactions have been recently shown to propagate prions using brain-derived or recombinant prion protein, but only in the presence of additional cofactors such as nucleic acids and lipids. Here, using a protein misfolding cyclic amplification variation, we show that prions causing such synthetic aggregates can be produced from rPrP by PMCA when seeded with mouse-derived RNA and acidic lipids (11). However, the presence of other mammalian cell-derived molecules in these preparations leaves open the question regarding the precise chemical identity of mammalian prions. It has also been reported that amyloid fibrils formed spontaneously from rPrP in the absence of any added cofactors can cause a transmissible neurological disorder in transgenic mice (12, 13). However, because the transgenic mouse used in these studies overproduce PrP^C (4—32-fold when compared with wild-type mice), it is unclear whether these synthetic fibrils indeed carry bona fide infectivity or, rather, accelerate a condition to which transgenic mice could potentially be predisposed due to the high level of PrP^C or other factors (14). This question is especially pressing because numerous attempts to infect wild-type mice or hamsters with similar material have not been successful.

We have shown recently that structurally distinct fibrillar aggregates can be produced from rPrP by PMCA when seeded with scrapie-derived brain homogenate or partially purified PrP^Sc (15, 16). Importantly, these PrP^Sc-seeded aggregates (denoted rPrP^PMCA or rPrP-res^Sc) display a proteinase K (PK) digestion pattern that is more closely related to PrP^Sc than that of spontaneously formed rPrP fibrils (15, 16). Here, we report that these aggregates, prepared solely from highly purified recombinant hamster prion protein (rShPrP) in the apparent absence of any additional cofactors, are infectious to wild-type hamsters.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Recombinant Prion Proteins**

Recombinant Syrian hamster full-length prion protein (rShPrP-(23–231)) and its N-terminally truncated fragment (rShPrP-(90–231)) were expressed in *Escherichia coli* and purified as described previously (17). The proteins were pure as judged by SDS-PAGE criteria. All procedures were performed in a laboratory that was never exposed to prions. The proteins were stored frozen in 10 mM sodium acetate buffer (pH 4.0).

**Preparation of PrP27–30**—Isolation and purification of PrP27–30, the PK-resistant core of PrP^Sc, from brains of 263K humans and animals (1–5). The pathogenic process in these diseases is typically associated with conformational conversion of a normal (cellular) prion protein, PrP^C, to a misfolded form, PrP^Sc. The “protein-only” model asserts that this rogue PrP^Sc conformer itself represents the infectious prion agent, self-propagating by binding to PrP^C and inducing its conversion to the abnormal PrP^Sc isoform (6).

Although this protein-only model is consistent with substantial experimental data (1–5), unequivocal proof for the purely proteinaceous nature of the infectious TSE agent has been elusive. Prion infectivity has been propagated, and even initiated *de novo*, under cell-free conditions using “protein misfolding cyclic amplification” (PMCA) (7–9). These PMCA reactions involved successive rounds of incubation and sonication of crude brain homogenates (7, 9) or largely purified brain- or cell-derived PrP^Sc and poly(A) RNA (8, 10) and, most recently, bacterially expressed recombinant PrP (rPrP) mixed with mouse-derived RNA and acidic lipids (11). However, the presence of other mammalian cell-derived molecules in these preparations leaves open the question regarding the precise chemical identity of mammalian prions. It has also been reported that amyloid fibrils formed spontaneously from rPrP in the absence of any added cofactors can cause a transmissible neurological disease in transgenic mice (12, 13). However, because the transgenic mouse used in these studies overproduce PrP^C (4—32-fold when compared with wild-type mice), it is unclear whether these synthetic fibrils indeed carry bona fide infectivity or, rather, accelerate a condition to which transgenic mice could potentially be predisposed due to the high level of PrP^C or other factors (14). This question is especially pressing because numerous attempts to infect wild-type mice or hamsters with similar material have not been successful.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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3 The abbreviations used are: TSE, transmissible spongiform encephalopathy; PrP^C, normal cellular prion protein; PrP^Sc, disease-associated scrapie isoform of prion protein; PMCA, protein misfolding cyclic amplification; rPrP, recombinant rPrP; rShaPrP-(23–231), recombinant Syrian hamster full-length prion protein; rShaPrP-(90–231), N-terminally truncated fragment of the recombinant Syrian hamster prion protein; rShaPrP^PMCA, recombinant Syrian hamster PrP aggregates generated in PMCA reaction; PK, proteinase K; PrP27–30, PK-resistant core of PrP^Sc; GPI, glycosylphosphatidylinositol; PBS, phosphate-buffered saline.
scrapie-infected hamsters was performed by a modification of the method of Hilmert and Diringer (18) as described previously (19). The purified material was resuspended by sonication in Tris-buffered saline (pH 7.5) containing 0.1% sulfobetaine 3–14 and stored at −20 °C. The concentration of PrP27–30 was estimated by densitometric analysis of quantitative Western blots using full-length recombinant hamster PrP as a standard.

**PMCA Experiments**—PrPSc-seeded PMCA of the recombinant hamster prion protein was performed essentially as described previously (15). Briefly, monomeric rShaPrP-(23–231) or rShaPrP-(90–231) was diluted to a concentration of 0.05 mg/ml in a conversion buffer (phosphate-buffered saline (PBS) containing 0.1% SDS and 0.1% Triton X-100, pH 7.4). One hundred-microliter aliquots of this solution were placed in 0.2-ml PCR tubes, and 100 ng of purified PrP27–30 was added as a seed. The tubes were positioned in a rack placed on the holder of a microplate sonicator (Misonix, Model 3000MP), and the samples were subjected to nine rounds of PMCA reaction, each of them consisting of 18 cycles of 40-s sonication (40% potency of the sonicator) followed by a 1-h incubation at 37 °C. After each round, an aliquot of the amplified samples was taken and diluted 10-fold (second and third rounds) or 100-fold (rounds four to nine) into the conversion buffer containing monomeric protein (0.05 mg/ml) as a substrate. The final product was analyzed by 12% NuPAGE (Invitrogen) and Western blotting after PK treatment for 1 h at 37 °C (PK:rShaPrP ratio of 1:10, w/w), as described previously (16). Control “mock PMCA”-treated samples of PrP27–30 were prepared by subjecting PrP27–30 seed (100 ng) to the same PMCA procedure as described above but in the absence of any rShaPrP substrate. The work was done using disposable laboratory supplies and aerosol barrier tips. The bench, pipettes, and other equipment were cleaned frequently with NaOH or bleach. The sonicator probe, barrier tips. The bench, pipettes, and other equipment were

Infectivity Assays in Hamsters—For infectivity assays with rShaPrP(23–231)PMCA, the final PMCA product was harvested by centrifugation (20,000 × g, 30 min, 4 °C), washed twice with PBS, and resuspended in 1 ml of PBS by sonication at a concentration of ~300 µg/ml. In second passage experiments, 1% brain homogenates of rShaPrP(23–231)PMCA-inoculated hamsters were prepared in PBS. Four-to-six-week-old female Syrian golden hamsters were used in bioassays. After anesthetization with isoflurane, hamsters were inoculated intracerebrally with isoflurane and heating at 100 °C for 5 min. The samples were separated by 12% NuPAGE (Invitrogen) and electrotransferred onto nitrocellulose membranes (Invitrogen). Immunodetection of PrP was performed using a chemiluminescence-based detection scheme involving the anti-PrP monoclonal antibody 3F4 (21) (1:10,000 dilution) and secondary horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Pierce).

**Results and Discussion**

To test the infectivity of rPrP(23–231)PMCA fibrils, we performed nine rounds of PMCA using as a substrate recombinant Syrian hamster full-length PrP (rShaPrP-(23–231)) or the 90–231 fragment (rShaPrP-(90–231)). In the first round, the reaction was seeded with partially purified, PK-digested PrPSc (PrP27–30) derived from 263K scrapie-infected hamster brain. Each subsequent round was seeded with a 1/10 or 1/100 volume of PMCA product from the previous round (see “Experimental Procedures”). The cumulative 10–14 dilution of the original PrPSc seed theoretically made its concentration ~0.01 ag/ml or 0.2 molecules/ml, an amount ~102-fold below one LD50 (a dose lethal to 50% of intracerebrally inoculated hamsters).

Multiple independently prepared rShaPrP-(23–231)PMCA or rShaPrP-(90–231)PMCA preparations were harvested and inoculated intracerebrally into groups of hamsters. Remarkably, a fraction of inoculated animals in all but one group developed clinical signs of scrapie, although there was considerable variability in the incubation times (ranging from 119 to 401 days) and attack rates (Table 1). In the second passage experiments using brain extract from affected hamsters as the inocula, all hamsters became infected with an incubation time of 84 ± 1 and 75 ± 4 days for rShaPrP-(23–231)PMCA- and rShaPrP-(90–231)PMCA-derived material, respectively (Table 1).

In control experiments, hamsters were inoculated with either undiluted PrP27–30 seed or a 1014-fold dilution thereof (equivalent to cumulative serial PMCA dilution). As expected, animals inoculated with the undiluted PrP27–30 seed suc-
The lesions produced by rShaPrP-(90–231)PMCA were very similar to each other but were both significantly different from that of 263K scrapie-inoculated hamsters (Fig. 1B). These distinct lesion profiles found in rShaPrP-(90–231)PMCA-inoculated hamsters were preserved upon second passage (Fig. 2, A and B), indicating that rShaPrP-(90–231)PMCA represents a stable prion strain that is different from 263K scrapie. The overall pattern of PrPSc immunostaining in rShaPrP-(90–231)PMCA-inoculated hamsters was similar to hamsters inoculated with 263K scrapie. However, the PrPSc staining intensity was markedly lower in hamsters inoculated with rShaPrP-(90–231)PMCA, and this low staining intensity was maintained in the second passage (Fig. 2, C–E). Again, this points to distinct strain properties of rShaPrP-(90–231)PMCA, strongly arguing against the possibility that contamination could be responsible for the infectivity of the rPrP-(90–231)PMCA preparations. The apparent emergence of a new prion strain (that is, distinct from the 263K scrapie seed) in our PMCA reaction is puzzling, especially given the previous findings that PMCA can preserve strain characteristics of the seed (7, 8). The apparent lack of strain-specific fidelity in rPrP-(90–231)PMCA propagation as observed in the present study contrasts with the observations in studies with yeast prions. In the latter system, conformation-encoded strain properties of infectious amyloid fibrils prepared in vitro from recombinant yeast prion protein were fully preserved upon transfection into living cells, and this strain-specific fidelity was considered important for formally proving the concept of protein conformation-based inherit-

### TABLE 1
Summary of infectivity bioassays in hamsters

| Inoculum* | No. of TSE-positive animals/ no. of inoculated | Incubation time of TSE-positive animals, days* |
|-----------|-----------------------------------------------|-----------------------------------------------|
| rPrP-(seeded with PrP27–30) | | |
| rShaPrP-(23–231) (Experiment I) | 5/10 | 328 ± 113 (133, 323, 391, 392, 401) |
| rShaPrP-(23–231) (Experiment II) | 6/9 | 251 ± 112 (119, 166, 166, 345, 345, 365) |
| rShaPrP-(90–231) (Experiment I) | 7/9 | 185 ± 83 (129, 129, 143, 145, 188, 196, 362) |
| rShaPrP-(90–231) (Experiment II) | 6/6 | 162 ± 16 (141, 154, 154, 166, 166, 188) |
| rShaPrP-(90–231) (Experiment III) | 1/7 | 187 |
| rShaPrP-(90–231) (Experiment IV) | 0/6 | NA* |
| 2nd passage of rShaPrP-(23–231) (Experiment I) | 6/6 | 84 ± 1 |
| 2nd passage of rShaPrP-(90–231) (Experiment I) | 6/6 | 75 ± 4 |
| 2nd passage of rShaPrP-(90–231) (Experiment II) | 6/6 | 75 ± 5 |

**Negative controls**

| Sample | No. of TSE-positive animals/ no. of inoculated | Incubation time of TSE-positive animals, days* |
|--------|-----------------------------------------------|-----------------------------------------------|
| Diluted PrP27–30 seed | 0/9 | NA* |
| Mock PMCA of PrP27–30 alone | 0/6 | NA* |
| rShaPrP-(23–231) monomer | 0/9 | NA* |
| rShaPrP-(90–231) monomer | 0/6 | NA* |

**Positive control**

| Sample | No. of TSE-positive animals/ no. of inoculated | Incubation time of TSE-positive animals, days* |
|--------|-----------------------------------------------|-----------------------------------------------|
| Undiluted PrP27–30 seed | 9/9 | 85 ± 4 |

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* Fifty microliters of each inoculum were used. For inocula derived from rShaPrP, the concentration of protein was 300 μg/ml in each case. For second passage of the rPrP-(90–231)PMCA group, 1% brain homogenate was used. The concentration of undiluted PrP27–30 seed was 1 μg/ml.

* Animals showed clinical signs for TSE and were positive for PrPSc.

* Mean ± standard deviation.

* Bioassays were performed at Rocky Mountain Laboratories; all other bioassays were done at Case Western Reserve University.

* NA, not applicable. Animals in these groups did not show any neurological abnormalities during their life span and were not positive for PrPSc.

* Hamster sacrificed at 133 days after inoculation was used.

* Hamster sacrificed at 129 days after inoculation was used.

* Hamster sacrificed at 143 days after inoculation was used.

* Sample was prepared by serial 10−14 dilution of PrP27–30 seed.

* Sample was prepared by subjecting PrP27–30 alone (no rShaPrP substrate) to a mock PMCA procedure that results in 10−14 dilution of PrP27–30.

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Summary of infectivity bioassays in hamsters
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FIGURE 1. Characterization of PrPSc and brain histopathology of rShaPrP-(23–231)PMCA-inoculated and control hamsters. **A**, Western blot analysis of PrPSc in brain homogenates from hamsters inoculated with PrP27–30 (lane 1), PrP27–30 seed after 10–14 dilution (lane 2), PrP27–30 seed subjected to mock PMCA (resulting in 10–14 dilution) in the absence of any rShaPrP substrate (lane 3), rShaPrP-(23–231)PMCA (lane 4), rShaPrP-(90–231)PMCA (lane 5), brain homogenate from rShaPrP-(23–231)PMCA-inoculated hamster (lane 6), and brain homogenate from rShaPrP-(90–231)PMCA-inoculated hamster (lane 7). Samples were analyzed before (−) and after (+) PK digestion (100 μg/ml, 1 h at 37 °C). The blot was probed with anti-PrP 3F4 antibody (1:10,000). **B**, lesion profiles in brains of hamsters inoculated with 263K scrapie PrP27–30 (red circle, n = 3), rShaPrP-(23–231)PMCA (blue triangle, n = 10), and rShaPrP-(90–231)PMCA (green square, n = 5). The profiles for hamsters inoculated with rShaPrP-(23–231)PMCA and rShaPrP-(90–231)PMCA were very similar to each other, but both were significantly different from that of hamsters inoculated with 263K scrapie PrP27–30. Specific brain regions showing statistically significant differences between hamsters inoculated with 263K scrapie PrP27–30 and rShaPrP-(90–231)PMCA are indicated by an asterisk (p < 0.001–0.04). For rShaPrP-(23–231)PMCA-inoculated hamsters, statistically significant differences (p < 0.001–0.02) from 263K-inoculated hamsters were observed in the same regions except for the thalamus. Symbols used for brain regions are: Cx, cerebral cortex; HI, hippocampus; Sub, subiculum; BG, basal ganglia; TH, thalamus; CE, cerebellum; Sept n, septal nuclei; BS, brainstem. Error bars indicate S.E. Statistical significance was determined using a two-tailed Student’s t test. C–E, immunohistochemical staining of PrPSc in the white matter of corpus callosum (CC) and gray matter of cerebral cortex (Cor). C, hamsters inoculated with 263K scrapie PrP27–30; D, first passage of rShaPrP-(90–231)PMCA; E, second passage of rShaPrP-(90–231)PMCA. The square in panel C indicates perivascular deposits of PrPSc, and arrows indicate plaque-like deposits. Bar = 100 μm.

FIGURE 2. Comparison of lesion profiles and PrPSc staining observed in first and second passage of rShaPrP-(23–231)PMCA prions. **A**, lesion profile in first (blue triangle, n = 10) and second (black open squares, n = 5) passage of rShaPrP-(23–231)PMCA; **B**, lesion profile in first (blue triangle, n = 5) and second (black open squares, n = 12) passage of rShaPrP-(90–231)PMCA. Lesion profiles for 263K scrapie-inoculated hamsters (red circle, n = 3) are shown for comparison. No statistically significant differences were found between profiles for first and second passages of both rShaPrP-(23–231)PMCA and rShaPrP-(90–231)PMCA (p > 0.05), but each of these profiles was significantly different from that for 263K scrapie (p < 0.00001–0.02). Symbols used for brain regions are: Cx, cerebral cortex; HI, hippocampus; Sub, subiculum; BG, basal ganglia; TH, thalamus; CE, cerebellum; Sept n, septal nuclei; BS, brainstem. Error bars indicate S.E. Statistical significance was determined using a two-tailed Student’s t test.

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ance in yeast (25, 26). Such perfect reproduction of strain-specific patterns in experiments with synthetic mammalian prions may require the use of pure PrP substrate that is chemically identical to brain PrPSc (i.e. with posttranslational modifications such as glycosylation and the GPI anchor).

Given the above caveat of studies with aggregates generated in vitro from bacterially expressed recombinant PrP, one cannot formally rule out the possibility that these preparations trigger infectious TSE disease by some unknown mechanism other than acting as a physical template for the conversion of brain PrPSc into the PrPSc state. However, the latter possibility is purely theoretical and highly unlikely.

The recently reported de novo generation of infectivity (i.e. in the absence of any preexisting PrPSc seed) from brain-derived PrPSc and poly(A) RNA (8), total brain homogenate (9), or recombinant PrP in the presence of mouse RNA and lipids (11) raises the question as to whether this could also be accomplished using highly purified rPrP in the absence of any cofactors. Our preliminary data suggest that infectious material can indeed be generated de novo from pure rShaPrP, although the probability of such an event appears to be very low. After inoculation is shown in panel F. Circles in panels D and E exemplify small clusters of typical vacuoles. Although 263K scrapie PrP27–30-inoculated hamsters showed widespread spongiform degeneration, the spongiform degeneration in rShaPrP-(90–231)PMCA-inoculated hamsters was much less severe, and this difference was maintained in the second passage. No spongiform degeneration was observed in control hamsters. Bar = 100 μm.
low. These experiments with de novo generated rPrP prions are still ongoing; after completion, they will be reported elsewhere.

Altogether, the present data demonstrate that infectious prions can be generated by PMCA from highly purified rPrP in the absence of any apparent cofactors. rPrP differs from typical brain-derived PrPSc by its lack of N-linked glycans and a GPI moeity, and these data confirm previous indications that these post-translational modifications are not essential for prion propagation in vivo (27, 28). Variable attack rates and long incubation times indicate that the infectivity of rShaPrP-PMCA is very low relative to brain-derived PrPSc. This is consistent with our observation that inoculation of hamsters with rShaPrP-(90–231)PMCA at ∼25-fold lower dose than that described in Table 1 did not cause any signs of TSE (data not shown). Although the reasons for much lower infectivity of our conversion product (per unit of protein) when compared with that of brain-derived PrPSc are at present unclear, this could be due to one of the following factors (or a combination thereof). (i) The infectious material might constitute only a small subfraction of our PMCA product; (ii) other cofactors that are supplied in vivo, and not in our scrapie-seeded rPrP-PMCA reactions, might promote the infectivity of the conversion product; (iii) non-infectious off-pathway products of the rPrP-PMCA reaction might interfere with the infectivity, or promote the clearance, of the infectious scrapie-seeded rPrP PMCA product. The complete attack rate and greatly reduced incubation period on second passage indicate that the artificial rShaPrP-PMCA prions adapt to hamsters, as takes place with archetypal prion diseases.

It was recently reported that material infectious to wild-type mice can be formed de novo from the mixture of mouse rPrP and relatively large quantities of mouse-derived RNA and an acidic lipid, phosphatidylglycerol (11). Some apparent infectivity was also reported to be associated with spontaneously formed recombinant PrP amyloid fibrils subjected to “annealing” by incubation with normal brain homogenate, although in this case, clinical signs were observed only in the second passage and only after very long incubation times (481–565 days) (29). In contrast to these recent studies on de novo generated infectivity, the infectious material in our work was generated in a PrPSc-seeded PMCA reaction and, most importantly, in the absence of any added cofactors. The finding that truly infectious prions causing clinical TSE disease in a wild-type host can be generated from pure bacterially expressed prion protein provides a strong support for the protein-only model of these neurological disorders. Furthermore, our data demonstrate that additional cofactors that have been used in previous studies (nucleic acids, lipids) are not obligatory for generation of infectious mammalian prions. However, given the apparent low infectivity titer of the material generated in the present study, it appears that these (and/or other) cofactors may enhance or accelerate prion protein misfolding to the infectious form in vitro as well as in vivo.

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