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In Vitro Generation of Infectious Scrapie Prions

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Summary

Prions are unconventional infectious agents responsible for transmissible spongiform encephalopathy (TSE) diseases. They are thought to be composed exclusively of the protease-resistant prion protein (PrP^{res}) that replicates in the body by inducing the misfolding of the cellular prion protein (PrP^{C}). Although compelling evidence supports this hypothesis, generation of infectious prion particles in vitro has not been convincingly demonstrated. Here we show that $PrP^{C} \rightarrow PrP^{res}$ conversion can be mimicked in vitro by cyclic amplification of protein misfolding, resulting in indefinite amplification of PrP^{res}. The in vitro-generated forms of PrP^{res} share similar biochemical and structural properties with PrP^{res} derived from sick brains. Inoculation of wild-type hamsters with in vitro-produced PrP^{res} led to a scrapie disease identical to the illness produced by brain infectious material. These findings demonstrate that prions can be generated in vitro and provide strong evidence in support of the protein-only hypothesis of prion transmission.

Introduction

Prion diseases, also called transmissible spongiform encephalopathies (TSEs), are some of the most intriguing infectious disorders affecting the brains of humans and animals. The group is comprised of Creutzfeldt-Jakob disease (CJD), kuru, Gerstmann-Sträussler Sheinker syndrome (GSS), and fatal familial insomnia (FFI) in humans and scrapie, bovine spongiform encephalopathy (BSE), and chronic wasting disease (CWD) in animals (Collinge, 2001). TSEs can have sporadic, inherited, and infectious origins. The nature of the transmissible agent has been the subject of intense debate (Prusiner, 1998; Mestel, 1996; Chesebro, 1998; Soto and Castilla, 2004). Initially the agent was thought to be a slow virus (Sigurdsson, 1954), but further research has indicated that the infectious particle is significantly different from viruses and other conventional agents (Prusiner, 1998). Accordingly, a new hypothesis regarding the nature of the transmissible agent has emerged and recently gained widespread acceptance. This hypothesis proposes that the material responsible for the disease transmission is uniquely composed of a protein that has the surprising ability to replicate itself within the body (Prusiner, 1982; Prusiner, 1998). This newly discovered pathogen is called a proteinaceous infectious particle, or prion (Prusiner, 1982).

An important step in understanding the nature of this novel infectious agent was the isolation of the protease-resistant prion protein (PrP^{res}) from the infectious material (Bolton et al., 1982). Using biochemical and immunological methods, it was shown that PrP^{Sc} and infectivity copurified and that the concentration of the protein was proportional to the infectivity titer (Gabizon et al., 1988). Infectivity was retained in highly purified preparations of PrP^{res}, in which no other component is detectable. In addition, infectivity was convincingly reduced by agents that destroy protein structure and by anti-PrP antibodies (Gabizon et al., 1988). Purification of PrP led to the identification of the gene containing the sequence that encodes PrP (Basler et al., 1986). PrP mRNA is the product of a single gene that is synthesized in the brains of healthy animals and is also constitutively expressed in many cell types (Basler et al., 1986). Thus, PrP has two alternate forms: the normal cellular protein (PrP^{C}) and the pathological isoform (PrP^{res}). Chemical differences between the PrP^{C} and PrP^{res} isoforms have not been detected (Stahl et al., 1993), and the conversion seems to involve a conformational change in which the α-helical content of the normal protein is reduced while the β sheet content is increased (Pan et al., 1993). Structural changes are accompanied by alterations in the biochemical properties (Prusiner, 1998; Cohen and Prusiner, 1998). As such, PrP^{C} is soluble in nondenaturing detergents while PrP^{res} is insoluble, and PrP^{C} is readily digested by proteases while PrP^{res} is mostly resistant, resulting in the formation of an N-terminally truncated fragment known as PrP27–30.

The structural conversion of PrP^{C} into PrP^{res} catalyzed by PrP^{res} has been done in vitro. The cell-free conversion system developed by Caughey and coworkers (Kocisko et al., 1994) using purified PrP^{C} mixed with stoichiometric amounts of purified PrP^{res} produced a low yield of PrP^{res} formation under nonphysiological conditions, making it difficult to evaluate the infectious properties of in vitro-produced misfolded protein. However, the fact that PrP^{res} was able to induce the transformation of the normal protein into more of itself represented important evidence in favor of the prion hypothesis. More recently, we developed a new in vitro conversion system to convert large quantities of PrP^{C} into PrP^{res} using minute amounts of PrP^{res} (Saborio et al., 2001). This system, called PMCA (protein misfolding cyclic amplification), confirms a central facet of the prion hypothesis, which is that prion replication is a cyclical process and that newly produced PrP^{res} can further propagate the protein misfolding (Soto et al., 2002).

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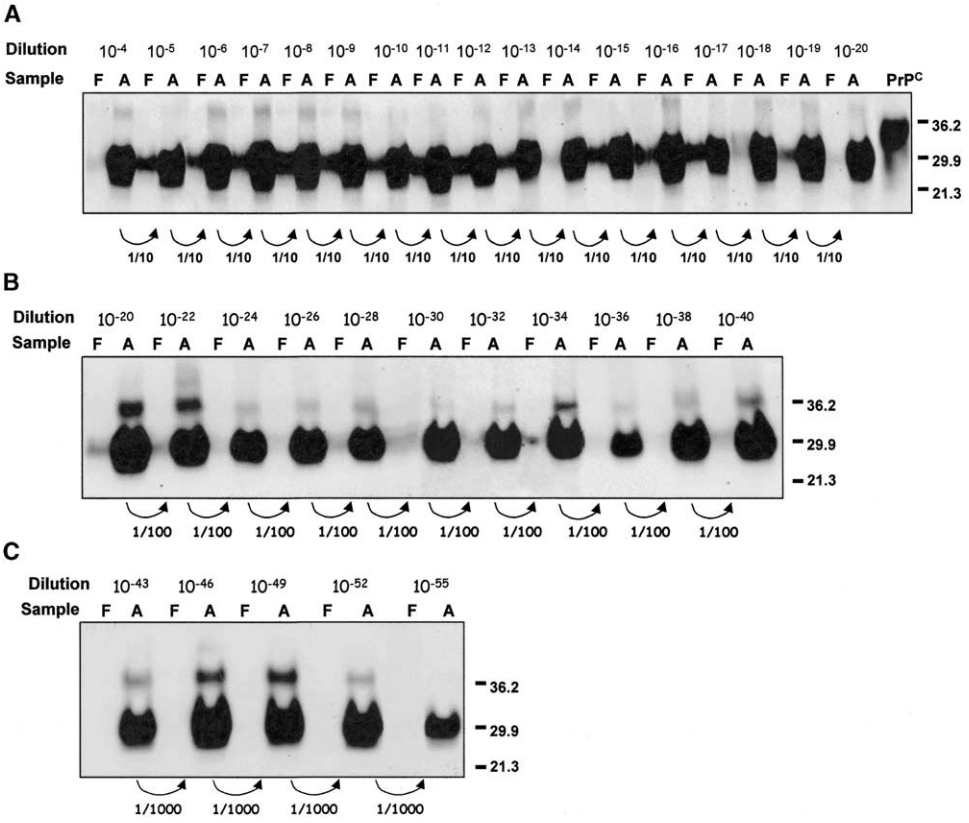


Figure 1. Infinite PrP^{res} Replication In Vitro by Serial PMCA

(A) Hamster scrapie brain homogenate was diluted 10^4-fold into normal brain homogenate and subjected to 20 cycles of PMCA as described in Experimental Procedures. The amplified material was diluted 10-fold into normal brain homogenate and amplified again. This procedure was repeated several times to reach a 10^{-20} dilution of scrapie brain homogenate. Mathematical estimations indicate that the last molecule of PrP^{res} from the inoculum was lost at the 10^{-14} dilution.

(B) PrP^{res} samples from the 10^{-20} dilution were further replicated up to 10^{-40} by serial PMCA after 100-fold dilutions followed by 48 PMCA cycles.

(C) PrP^{res} samples from the 10^{-40} dilution were further replicated to reach a 10^{-55} dilution by performing 48 PMCA cycles and 1000-fold dilutions of the samples. F, frozen samples; A, amplified samples.

The PMCA technology was experimentally designed to mimic some of the fundamental steps involved in PrP^{res} replication in vivo at an accelerated rate (Soto et al., 2002). In a cyclic manner conceptually analogous to PCR cycling, a minute quantity of PrP^{res} is incubated with excess PrP^{C} to enlarge the PrP^{res} aggregates that are then sonicated to generate multiple smaller units for the continued formation of new PrP^{res} (Saborio et al., 2001). A modest level of amplification has also been observed without sonication (Saborio et al., 2001; Lucassen et al., 2003; Deleault et al., 2003), and the extent of conversion depends upon the number of PMCA cycles (Saborio et al., 2001; Bieschke et al., 2004; Piening et al., 2005).

In spite of the compelling evidence supporting the prion hypothesis, there is still considerable skepticism among scientists (Chesebro, 1998; Mestel, 1996; Soto and Castilla, 2004). It is widely accepted that the prion hypothesis can ultimately only be proven once infectivity can be generated in a test tube. If the infectious agent is misfolded PrP^{res} and its replication is promoted by interaction with PrP^{C}, then it should be possible to reproduce the whole process entirely in vitro. Several strategies have been employed to achieve this aim, but all have failed or have produced infectivity after a very long time of incubation and only in transgenic animals highly overexpressing mutated or truncated versions of PrP (May et al., 2004; Soto and Castilla, 2004; Legname et al., 2004).

The aim of this study was to evaluate the biochemical, structural, and infectious properties of in vitro-generated PrP^{res}. For this purpose we have optimized the PMCA procedure to produce high levels of PrP^{res} formation in vitro and developed the conditions to maintain PrP^{res} indefinitely propagating in vitro at the expense of PrP^{C} with the goal of producing misfolded protein free from brain PrP^{res} inoculum. Our results indicate that PrP^{res} generated in vitro under conditions in which no brain-derived PrP^{res} is present in the sample has the same structural and biochemical properties as the disease-associated protein. More importantly, we have shown that the PMCA-generated protein is infectious to wild-type animals, leading to a disease that has clinical, histological, and biochemical properties that

are identical to the illness produced by the prion infectious agent. These findings represent the first time that wild-type animals have been infected with PrP^{res} generated in vitro and thus constitute some of the strongest evidence yet available supporting the prion hypothesis.

Results

Infinite Replication of PrP^{res} In Vitro

Hamster brains infected with 263K scrapie were homogenized and diluted 10^4-fold into a 10% normal hamster brain homogenate. Samples were either immediately frozen or subjected to 20 PMCA cycles. After this first round of PMCA, a small aliquot of the amplified samples was taken and diluted 10-fold into more normal brain homogenate. These samples were again immediately frozen or amplified by 20 PMCA cycles. This procedure was repeated several times, and PrP^{res} generation was determined by Western blot after proteinase K (PK) digestion to remove the remaining PrP^{C}. Figure 1A shows the results of an experiment in which 17 rounds of PMCA were performed. In the final series of PMCA, the amount of scrapie brain homogenate is equivalent to a 10^{20}-fold dilution. Estimation of the amount of PrP^{res} inoculum present indicates that after this dilution no molecules of brain-derived protein were present, whereas the amount of newly generated PrP^{res} corresponds to approximately 1×10^{11} molecules. The amplified samples for the 10^{-20} dilution were further diluted and subjected to several rounds of PMCA separated by 100-fold dilutions to reach a final dilution of scrapie brain homogenate equivalent to 10^{-40} (Figure 1B). The serial replication of PrP^{res} was additionally continued up to a 10^{-55} dilution by performing a series of 1000-fold dilutions followed by 48 cycles of PMCA (Figure 1C). We conclude from these results that PMCA enables an infinite replication of PrP^{res} in vitro. Interestingly, the signal can be fully recovered even after 1000-fold dilution of the sample, suggesting that the amplification rate is at least 1000. Moreover, the rate of PrP replication was not altered upon dilution, which suggests that newly converted protein is capable of inducing PrP^{res} formation with an efficiency similar to that of brain-derived PrP^{res}. A control experiment in which the healthy brain homogenate was serially diluted into itself and subjected to the same number of PMCA cycles as described above but in the absence of PrP^{res} inoculum did not show any protease-resistant PrP under any conditions (data not shown).

Biochemical and Structural Properties of In Vitro-Generated PrP^{res}

An in vitro-generated PrP^{res} sample that does not contain any brain-derived PrP^{res} provides an ideal material for analyzing the biochemical and structural properties of the in vitro-produced protein and comparing them with the properties of in vivo-generated PrP^{res}. A first comparison using Western blot profiles indicates that in vitro replication leads to a protein with identical electrophoretic mobility and glycosylation pattern to the disease-associated misfolded protein (Figure 2A). Indeed, experiments using PrP^{res} inoculum from different species/strains with distinct Western blot profiles showed that newly generated PrP^{res} always follows the pattern of the misfolded protein used as template (Soto et al., 2005). Furthermore, amino acid composition analysis of highly purified PrP^{res} produced in vitro shows very similar results to those found using brain-derived PrP^{res} (data not shown), indicating that the cleavage site after PK digestion is the same in both proteins. This is important because PrP^{res} from different strains has been shown to have a distinct PK cleavage site due to the different folding or aggregation of the protein (Chen et al., 2000; Collinge et al., 1996). The similar glycosylation pattern of newly generated and brain-derived PrP^{res} was further confirmed in experiments in which the proteins were treated with endoglycosidase (Figure 2A). The results demonstrate that the enzymatic removal of glycosylated chains occurs with similar efficiency in both proteins and that the unglycosylated bands have the same molecular weight.

A typical feature of misfolded PrP that has been extensively used to distinguish it from the normal protein isoform is the high resistance of the pathological protein to protease degradation. To compare the protease-resistance profile, similar quantities of PMCA-generated PrP^{res} (produced after a 10^{-20} dilution of scrapie brain homogenate) and brain-derived PrP^{res} were treated for 60 min with 50, 100, 150, 200, 250, 1,000, 2,500, 5,000 and 10,000 μg/ml of PK (Figure 2B). Both proteins were highly resistant to these large PK concentrations, and, strikingly, the pattern of resistance was virtually identical. This result is very significant because protease resistance is one of the hallmark properties of disease-associated PrP, and its quantity correlates tightly with infectivity (McKinley et al., 1983). Several procedures have been reported to produce protease-resistant forms of PrP, but in most of these cases the protease resistance was only detected at low concentrations of the enzyme and was thus not comparable to the extent of protease resistance seen in bona fide PrP^{res} (Jackson et al., 1999; Lehmann and Harris, 1996; Lee and Eisenberg, 2003).

Another typical property of misfolded PrP is its high insolubility in nonionic detergents. More than 95% of PrP^{res} derived both from brain and from PMCA was detected in the pellet after incubation and centrifugation in the presence of 10% sarkosyl, indicating that the two proteins are highly and similarly insoluble (Figure 3A). Insolubility of PrP^{res} was lost when the proteins were treated with >2 M guanidine hydrochloride, indicating that PrP^{res} from both origins was equally sensitive to denaturation by a chaotropic agent (Figure 3B).

The main difference between PrP^{C} and PrP^{res}, which is responsible for the other biochemical distinctions, is the secondary structure of the two proteins: whereas PrP^{C} is mainly α-helical, PrP^{res} is rich in β sheet conformation (Pan et al., 1993; Cohen and Prusiner, 1998). To study the secondary structure, PrP^{res} was highly purified from the brain of scrapie-sick hamsters or from samples amplified after a 10^{-20} dilution, as described in Figure 1. The standard purification procedure based on differential precipitation in detergents and protease degradation was used, and purity was estimated to be

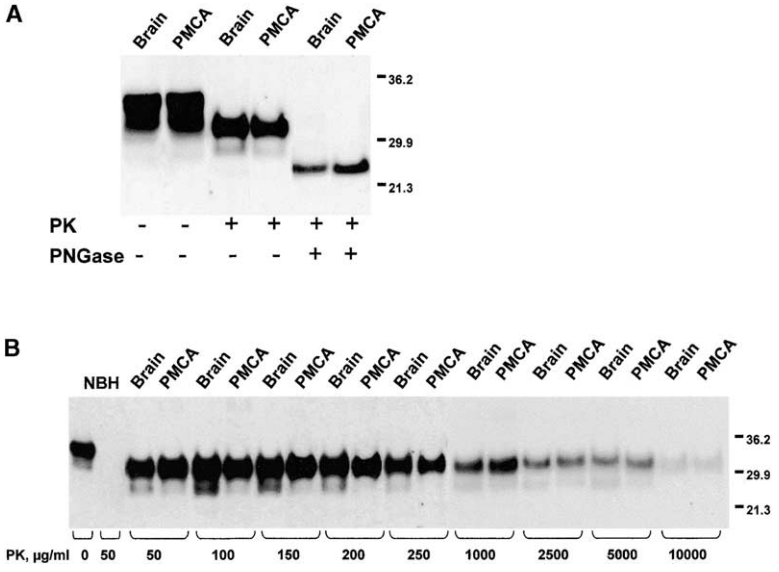


Figure 2. Electrophoretic Pattern, Deglycosylation, and Protease-Resistance Properties of In Vitro-Generated PrP^{res}

(A) Aliquots containing similar quantities of brain-derived PrP^{res} and the misfolded protein obtained by PMCA after a 10^{-20} dilution of scrapie brain homogenate (as shown in Figure 1A) were subjected to proteinase K (PK) digestion (50 µg/ml for 60 min) and loaded onto SDS-PAGE. Immunoreactive bands were observed using Western blot and a 3F4 monoclonal antibody. Similar aliquots of both proteins were subjected to deglycosylation by treatment with peptide N-glycosidase F for 2 hr at 37°C. Western blot analyses of different dilutions before and after deglycosylation were performed.

(B) Aliquots of PrP^{res} generated in vitro after a 10^{-20} dilution of brain PrP^{res} or misfolded protein obtained from scrapie brain homogenate were incubated for 60 min at 45°C, with the indicated concentrations of PK and PrP^{res} signal being detected by Western blot analysis as described in Experimental Procedures.

>90% by silver staining after electrophoresis and by amino acid composition analysis. Structural studies conducted using Fourier transform infrared (FTIR) spectroscopy of in vitro-generated PrP^{res} showed a spectrum consisting of high levels of β sheet content that was very similar to the spectrum obtained for purified brain-derived PrP^{res} (Figure 4). Deconvolution and fitting analysis of the spectra showed a virtually identical profile of secondary structures for both proteins (Figure 4), which are consistent with those previously reported for hamster PrP^{res} (Caughey et al., 1998; Pan et al., 1993). Importantly, the spectra show a still relatively high content of α-helical structure, as expected for disease-associated misfolded prion protein (May et al., 2004). The lack of α-helical structure is considered a drawback for most of the in vitro PrP refolding assays in which the PrP^{res}-like form is almost entirely organized in an aggregated β sheet structure (May et al., 2004). The high levels of β sheet structure, as well as the presence of random coil and α helix for PMCA-generated PrP^{res}, were also confirmed by circular dichroism studies. FTIR spectra of recombinant full-length hamster PrP^{C} produced in bacteria showed the expected high proportion of α helix and random coil and <10% of β sheet structure (data not shown).

The high content of β sheet structure of PrP^{res} results in a high tendency to form larger-order aggregates in vitro and in vivo (Prusiner et al., 1983; Ghetti et al., 1996). To study the ultrastructural characteristics of the aggregates, samples from highly purified brain-derived and PMCA-generated PrP^{res} were analyzed by electron microscopy after negative staining. Both proteins make

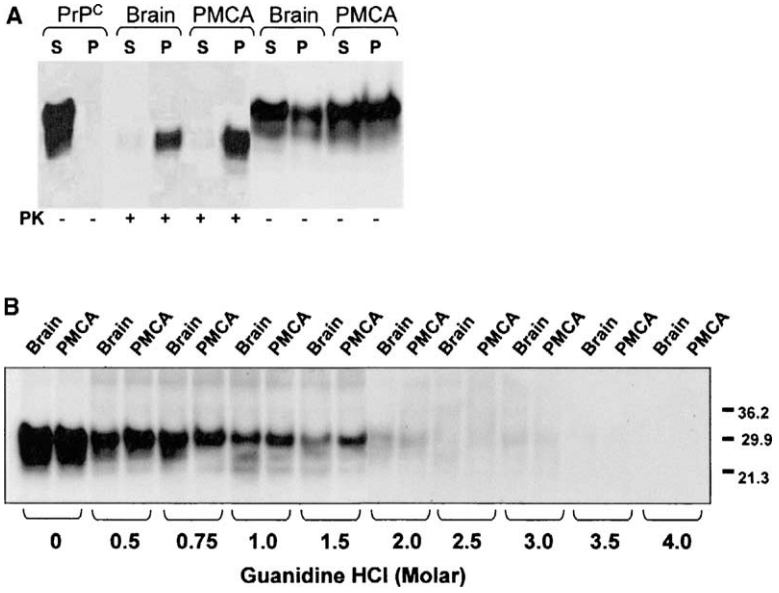
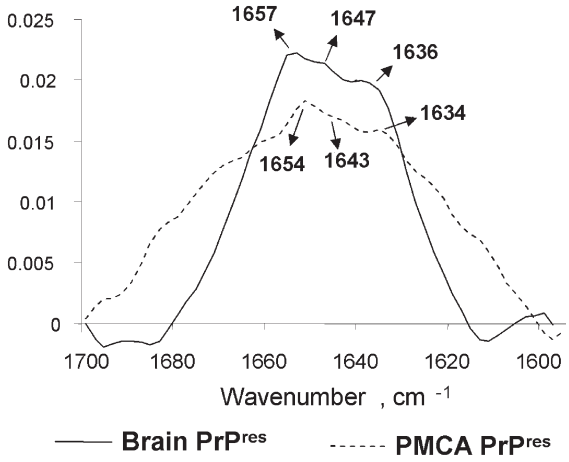


Figure 3. Detergent Solubility Studies

(A) Samples containing PMCA-generated or brain-derived PrP^{res} in 10% sarkosyl (final concentration) were centrifuged at 100,000 × g for 1 hr at 4°C, and the pellet (P) and supernatant (S) were treated with PK. PrP signal was detected using Western blotting. The distribution of PrP^{C} not treated with PK is shown as a control.

(B) Samples of PrP^{res} produced in vitro and in vivo were treated with the indicated concentrations of guanidine hydrochloride for 2 hr and thereafter centrifuged as described earlier. PrP^{res} in the pellet was detected by Western blot after PK digestion.



Secondary structure	Brain PrP^{res}	PMCA PrP^{res}
α-helix (1652-1657 cm^{-1})	36	36
β-sheet (1632-1636 cm^{-1})	31	32
Unordered (1642-1648 cm^{-1})	33	32

Figure 4. Secondary Structure of PMCA-Generated and Brain-Derived PrP^{res}

Samples of highly purified PrP27–30 (5 mg/ml in phosphate-buffered saline) obtained from PrP^{res} generated in vitro after a 10^{-20} dilution of brain PrP^{res} or from 263K scrapie hamster brain were loaded into Fourier transform infrared spectroscopy cuvettes and dried by passing a flow of nitrogen. Fourier self-deconvolution and curve fitting of the amide I region of the spectra was done using the Grams software. The table shows the estimation of the percentage of different secondary-structure motifs.

typical prion rod-like structures that are 10 to 20 nm in diameter and 50 to 100 nm in length (Figure S1), as previously described (Prusiner et al., 1983; Wille et al., 2000).

A hallmark property of prions is their capability to sustain autocatalytic replication in vivo (Prusiner, 1998). Injection of brain extracts containing PrP^{res} into an animal can further direct the conversion of normal PrP^{C}, and the misfolded protein can in this way keep replicating across animals and generations (Prusiner, 1998). The results shown in Figure 1 suggest that newly formed PrP^{res} is able to maintain replication in vitro even in the absence of brain-derived PrP^{res}. However, in order to analyze whether the efficiency of conversion is the same, we compared the rate of PrP^{C} conversion induced by brain-derived and PMCA-produced PrP^{res}. For these experiments, aliquots of both samples containing a similar amount of PrP^{res} equivalent to a 100-fold dilution of scrapie brain homogenate were further diluted into normal brain homogenate and subjected to 20 amplification cycles. As shown in Figure 5A, both samples were able to convert high levels of PrP^{C} to produce a similar amount of PrP^{res}. The efficient conversion was lost under these conditions when the samples were diluted more than 160-fold (16,000-fold in total). This result indicates that an approximately 300-fold amplification rate was obtained for both brain and PMCA PrP^{res} using 20 amplification cycles (Figure 5A). As described before, the rate of amplification depends upon the number of cycles performed (for example, a >6500-fold amplification was obtained when samples were subjected to 140 cycles), but, again, this rate was similar regardless of whether PrP^{res} came from in vivo brain samples or from in vitro-produced protein (data not shown).

Finally, we studied whether the converting activity of in vitro-generated PrP^{res} is as resistant to denaturation as has been reported for brain PrP^{res}. Samples of brain-derived and in vitro-generated PrP^{res} were subjected to thermal denaturation by incubation at 100°C, 110°C, 120°C, and 140°C for 1 hr. Thereafter, these samples were used to trigger PrP^{res} formation by diluting them into normal brain homogenate and performing 20 PMCA cycles. Generation of new PrP^{res} was not altered by previously heating the samples at 100°C or 110°C, but this activity was dramatically reduced by incubating PrP^{res} at 120°C and completely abolished after heating at 140°C (Figure 5B). Interestingly, the heat-resistance profile of both brain-derived and PMCA-produced PrP^{res} was very similar, further supporting the hypothesis that the two forms resemble each other.

PrP^{res} Generated In Vitro Is Infectious

One objective that has long been pursued is the in vitro production of prion infectious material by inducing the misfolding of PrP. Successful completion of this experiment is widely regarded as the final proof for the controversial protein-only hypothesis of prion propagation (Soto and Castilla, 2004). The serial replication of PrP^{res} in vitro by PMCA provides a perfect system to achieve this aim because, after many rounds of amplification following serial dilution of PrP^{res} inoculum, we were able to produce a preparation of misfolded protein that was biochemically and structurally identical to brain-derived PrP^{res} but lacked any molecules of the initial scrapie-infected inoculum. To determine the infectious capability of in vitro-generated PrP^{res}, groups of wild-type Syrian hamsters were inoculated intracerebrally (i.c.) with samples generated by 6 or 16 rounds of serial PMCA separated by 10-fold dilutions as described in Figure 1 (see Figure S2 for a schematic description of the groups used in this study). Since at the first PMCA the dilution of scrapie brain homogenate was 10^{-4}, the final dilution of scrapie material in these groups corresponds to 10^{-9} and 10^{-19}, respectively. An additional 10-fold dilution in phosphate-buffered saline was performed in all samples before inoculation. As shown in Figure 1, despite the large dilution, the quantity of PrP^{res} remained constant after amplification. Detailed estimation by quantitative Western blot analysis indicated that the amount of PrP^{res} in these samples was similar to the quantity of PK-resistant protein present in a 10^{-4} dilution of 263K hamster brain, which contains approximately 10^{10} molecules of the misfolded protein.

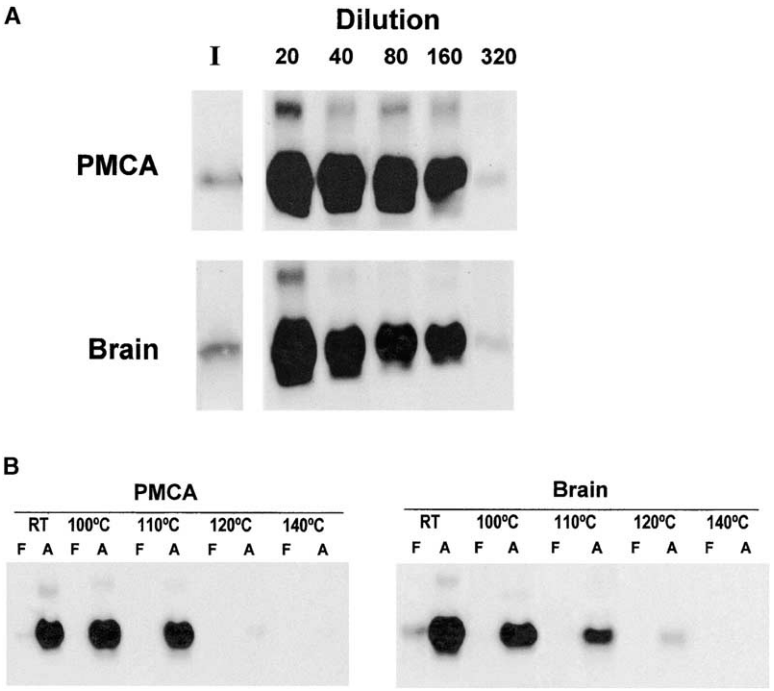


Figure 5. Self-Propagation and Heat Inactivation of PrP^{res}

(A) The efficiency with which PMCA-generated PrP^{res} induced the conversion of PrP^{C} in vitro was compared to the rate of conversion induced by brain-derived PrP^{res}. For this purpose, small and equivalent aliquots of both proteins (lane 1, I) were diluted 20-, 40-, 80-, 160-, and 320-fold into normal brain homogenate and subjected to 20 cycles of PMCA. The formation of newly generated PrP^{res} was determined using Western blot analysis.

(B) The resistance of PrP^{res} from both origins to heat inactivation was determined by incubating equivalent aliquots of the proteins at the indicated temperatures for 20 min. Thereafter, the samples were diluted 100-fold into normal brain homogenate and tested for their ability to induce the conversion of normal PrP^{C} into the protease-resistant isoform using 20 cycles of PMCA. F: frozen samples; A: amplified samples.

In the animals infected with the 10^{-10} dilution (group 6 in Table 1), 99.99999% of this material corresponded to newly generated PrP^{res} (0.99999×10^{10} molecules) and only 0.000001% to brain-derived PrP^{res} (1×10^{4} molecules). In the 10^{-20} dilution (group 7), 100% of PrP^{res} was newly generated protein (1×10^{10} molecules). Strikingly, all of the animals in these two sets (groups 6 and 7) showed typical signs of scrapie and died of the disease at around 170 days after inoculation (Table 1).

Based on our experience with the 263K experimental model and the literature reports, the 10^{-9} dilution of scrapie brain homogenate is the last dilution in which infectivity is observed (and only in some animals). Therefore, we hypothesized that dilutions equivalent to 10^{-10} and 10^{-20} would not produce any detectable disease (Table 1). This estimation was confirmed by the results obtained in our control groups (Table 1; groups 1, 2, 3, and 4). Two different negative control groups

Table 1. Infectivity Associated with In Vitro-Generated PrP^{res}

Group	Experimental Condition	Scrapie Brain Dilution	Molecules of PrP^{res} (Brain/PMCA)	Predicted Survival Time (% Sick Animals)	Observed Survival Time (Number of Sick/Total Animals)
1	Negative control: SBH diluted in NBH nonamplified	10^{-10}	$\sim 10^{4}$ (10^{4}/0)	>600 days (0%)	>400 days (0/6)
2	Negative control: SBH diluted in NBH nonamplified	10^{-20}	0 (0/0)	>600 days (0%)	>400 days (0/6)
3	Negative control: SBH diluted in null BH amplified	10^{-10}	$\sim 10^{4}$ (10^{4}/0)	>600 days (0%)	>400 days (0/6)
4	Negative control: SBH diluted in null BH amplified	10^{-20}	0 (0/0)	>600 days (0%)	>400 days (0/6)
5	Positive control: SBH diluted in NBH nonamplified	10^{-4}	$\sim 10^{10}$ (10^{10}/0)	94 ± 2.7 days (100%)	106 ± 2.9 days (6/6)
6	Experiment: SBH diluted in NBH amplified	10^{-10}	$\sim 10^{10}$ (10^{4}/0.99×10^{10})	?	177 ± 7.3 days (6/6)
7	Experiment: SBH diluted in NBH amplified	10^{-20}	$\sim 10^{10}$ (0/10^{10})	?	162 ± 3.5 days (6/6)

The number of molecules of PrP^{res} were estimated based on quantitative Western blot, using known concentrations of recombinant hamster PrP as standard. The predicted survival times are based on our previous data as well as published observations. Observed survival time is expressed as average ± standard error.

were used: the first one contained 10^{-10} and 10^{-20} dilutions of the scrapie brain homogenate into normal hamster brain homogenate done in serial 10-fold dilutions in parallel to the samples for PMCA but kept frozen without amplification (groups 1 and 2). The second control consisted of the scrapie brain homogenate diluted serially into PrP-knockout mouse brain homogenate up to 10^{10}- and 10^{20}-fold dilutions and subjected to the PMCA cycling (groups 3 and 4) in the same way as the study samples. None of the animals in these four groups of negative control samples had shown any signs of disease up to 400 days after infection (Table 1). This result clearly indicates that infectivity seen in the PMCA-amplified samples is associated with newly in vitro-generated PrP^{res}.

To compare the infectious capacity of PMCA-produced PrP^{res} with brain-derived infectivity, a group of animals were inoculated with a sample containing a similar amount of PrP^{res} as the ones produced after 6 and 16 serial PMCA rounds. As mentioned above, careful estimation using Western blot analysis showed that the quantity of PrP^{res} after the serial PMCA assays was equivalent to a 10^{-3} dilution of scrapie brain homogenate (10^{-4} considering the further 10-fold dilution prior to inoculation). A positive control group of animals (group 5) injected with this dilution of scrapie brain developed the disease with a mean survival time of 106 days (Table 1). The material for this experiment and the dilution used correspond exactly to the sample utilized to begin PMCA amplification, so it serves as the double control of the infectivity present in the sample prior to any dilution and amplification as well as the infectivity associated with this amount of PrP^{res}. The survival time was shorter than the one obtained with the equivalent quantity of PMCA-generated PrP^{res}, indicating that the in vitro-generated misfolded protein was significantly less infectious. We are currently doing infectivity titration studies to find out exactly how much lower the infectivity in the samples is, but based on the survival time, in vitro-generated PrP^{res} seems to be between 10 and 100 times less infectious than the same quantity of brain-derived PrP^{res}.

The clinical signs observed in the disease produced by the amplified samples were identical to those of the animals inoculated with infectious brain material and included hyperactivity, motor impairment, head wobbling, muscle weakness, and weight loss. In order to evaluate whether the biochemical and neuropathological characteristics of the disease were also the same, we performed a detailed comparative study of the brains of animals affected by the disease induced by brain-derived PrP^{res} (group 5) and PMCA-generated PrP^{res} (groups 6 and 7). Brain samples from all the animals in these three groups contained a large and similar quantity of PrP^{res}, which has an identical glycosylation profile (Figure 6A). Conversely, no protease-resistant protein was detected in the brains of negative control animals. To further evaluate whether or not PMCA-generated infectivity represents a new strain, we compared the electrophoretic mobility after PK treatment and the glycoform pattern of PrP^{res} with those of two other standard scrapie strains in hamsters, namely, 263K and drowsy. As shown in Figure 6B, whereas the Western blot pattern of the PMCA-generated PrP^{res} is identical to that of 263K (the strain used to produce new PrP^{res} by PMCA), it is substantially different from that of drowsy, a strain known to differ biochemically from 263K.

Histological analysis showed typical spongiform degeneration of the brain (Figure 6C), and samples from animals infected with in vitro-produced PrP^{res} showed a pattern and extent of vacuolation that was indistinguishable from those coming from the brains of hamsters inoculated with infectious brain material (Figure S3). The same similarities were also seen when tissue samples were stained for PrP accumulation (Figure 6D) and astrogliosis (Figure 6E). Thus, based on all of the biochemical, histological, and clinical analyses of the animals, we concluded that in vitro-generated PrP^{res} triggers a similar neurological disorder as brain-derived PrP^{res}.

To evaluate whether infectious properties of in vitro-generated PrP^{res} were stable over time, serial transmission experiments were done. Brain from one of the animals inoculated with 10^{-20} dilution of scrapie brain homogenate subjected to 16 rounds of PMCA (group 7 in Table 1) was homogenized and injected intraperitoneally (i.p.) into a group of six wild-type hamsters. All animals inoculated with this material exhibited typical signs of the disease with a mean survival time of 136.5 days (Table 2). Hamsters inoculated i.p. with the same dilution of brain homogenate from an animal originally infected with brain-derived PrP^{res} (group 5 in Table 1) developed the disease at 100.2 days postinoculation (Table 2). No clinical signs were observed in negative control animals inoculated with normal brain homogenate. All animals in groups 2 and 3 of the experiment in Table 2 showed protease-resistant PrP in the brain after postmortem analysis (data not shown). These results indicate that the infectious agent generated in vitro is stable over time.

Discussion

Compelling evidence has accumulated over the last several years in support of the protein-only hypothesis of prion propagation (Soto and Castilla, 2004; Prusiner, 1998). However, the prion concept is still highly controversial, and it is widely accepted that the final proof for this hypothesis consists of the generation of infectious material in vitro by inducing the misfolding of the prion protein (Soto and Castilla, 2004). Although extensively attempted, the generation of infectious mammalian prions in vitro has been elusive. Paradoxically, de novo generation of infectious proteins has been successfully done in a simpler system in yeast. A yeast prion has been defined as an infectious protein that behaves as a non-Mendelian genetic element, which transmits biological information in the absence of nucleic acid. Diverse genetic, biochemical, and structural evidence has been provided in support of the prion nature of the yeast determinants Sup35p and Ure2p (for references, see Uptain and Lindquist, 2002). Recent studies showed that bacterially produced N-terminal fragments of Sup35p, when transformed into amyloid fibrils, were able to propagate the prion phenotype to yeast cells (King and Diaz-Avalos, 2004; Tanaka et al., 2004). Infec-

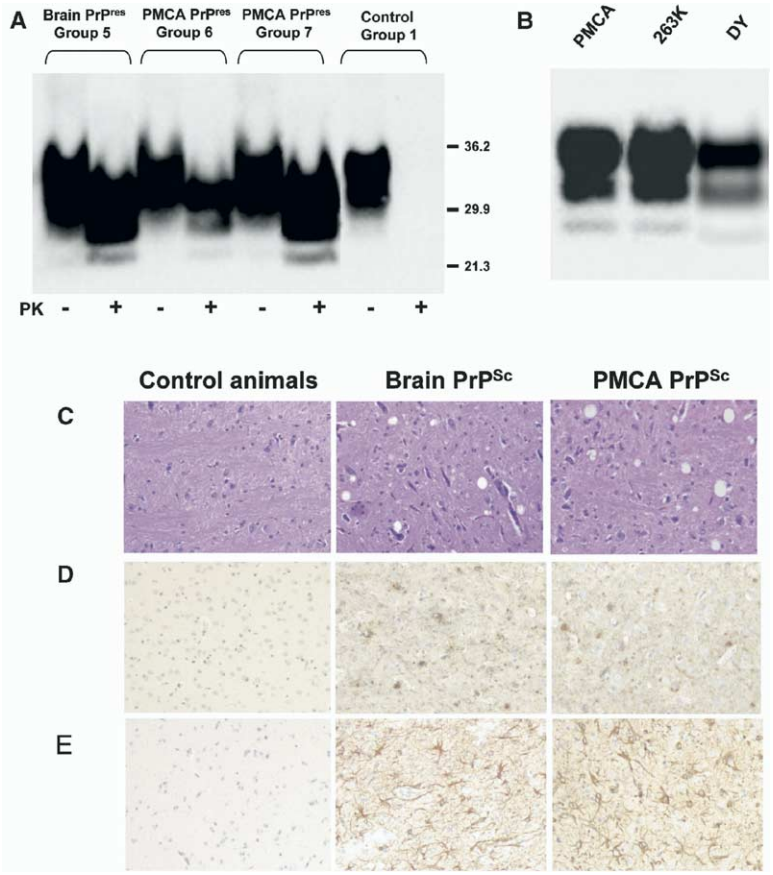


Figure 6. Biochemical and Histopathological Features of the Disease Induced by Inoculation with PMCA-Generated PrP^{res}

(A) The presence and quantity of PrP^{res} in the brains of animals in the terminal stage of the disease produced by inoculation with brain infectious material (group 5) or with the 10^{10}- and 10^{20}-fold diluted and amplified material (groups 6 and 7, respectively) were analyzed using Western blotting.

(B) The electrophoretic mobility and glycoform pattern of PrP^{res} obtained in the animals inoculated with PMCA-generated PrP^{res} were compared with the profile of 263K and drowsy (DY) strains of prions.

(C) Spongiform degeneration was evaluated after hematoxylin and eosin staining of medulla sections from symptomatic animals inoculated with either brain-derived or in vitro-produced PrP^{res}.

(D) PrP accumulation in these animals was evaluated by staining the tissue with the 3F4 anti-PrP monoclonal antibody as described in Experimental Procedures.

(E) Reactive astrogliosis in animals from both groups was evaluated by histological staining with glial fibrillary acidic protein antibodies.

For the results shown in this figure, all animals in groups 5–7 (Table 1) were analyzed, and the figure corresponds to a representative picture of these animals. As a control, brain samples from one animal in group 1 sacrificed without signs of the disease at 280 days after inoculation were used.

tion of yeast with different conformers led to generation of distinct [PSI^+] strains in vivo, indicating that differences in the conformation of the infectious protein determine prion strain variation.

Different strategies have been used to attempt the in vitro generation of mammalian prion infectious material (Soto and Castilla, 2004; May et al., 2004). An approach that has been explored for the purpose of generating de novo infectivity involved the production of PrP containing mutations associated with inherited TSEs. Several mutant PrP^{res}-like molecules have been generated, some of which have been shown to acquire various biochemical properties of PrP^{res}, but so far, none of them have been shown to be infectious (Lehmann and Harris, 1996; Chiesa et al., 1998). Another strategy consisted of inducing the misfolding of recombinant protein or short PrP synthetic peptides into β sheet-rich structures exhibiting some of the biochemical and biological properties of PrP^{res} (Jackson et al., 1999; Baskakov et al., 2000; Zhang et al., 1995; Lee and Eisenberg, 2003). The hope in these experiments was that infectivity might be generated even if a very small percentage of the protein altered in vitro adopted the "infectious folding." Unfortunately, these experiments have largely failed. Very recently, however, a recombinant mouse PrP fragment (residues 89–230) assembled into amyloid fibrils was found to induce a TSE-like disease with PrP^{res} formation when injected in transgenic mice overexpressing the same PrP sequence (Legname et al., 2004). The disease was later transmitted to wild-type animals in a second passage. These findings have come close to being the long-awaited definitive proof

Table 2. Secondary Transmission of In Vitro-Generated PrP^{res}

Group	Experimental Condition	Scrapie Brain Dilution	Survival Time (Number of Sick/Total Animals)
1	Negative control NBH	2×10^{-2}	>200 days (0/6)
2	Positive control[a]: animals infected with brain PrP^{res}	2×10^{-2}	100.2 ± 7.7 days (6/6)
3	Experiment[b]: animals infected with PMCA PrP^{res}	2×10^{-2}	136.5 ± 12.9 days (6/6)

The quantity of PrP^{res} inoculated in animals in groups 2 and 3 was similar as evaluated by Western blot. Survival time is expressed as average ± standard error.

[a] Animals were inoculated i.p. with 200 μl of a 1:50 dilution of brain homogenate from one animal in group 5 in Table 1.

[b] Animals were inoculated i.p. with 200 μl of a 1:50 dilution of brain homogenate from one animal in group 7 in Table 1.

of the prion hypothesis, but some experimental caveats remain to be addressed. The fact that the disease was originally transmitted to transgenic animals largely overexpressing the PrP gene and not to wild-type animals is a matter of concern because it is well known that this type of animals develops a prion-like disease spontaneously (Westaway et al., 1994; Castilla et al., 2004a; Chiesa et al., 1998). In other words, the effect seen might just be an acceleration of the disease process that was set to occur spontaneously at a later time. In this sense, an important missing control was the inoculation of animals in a second passage with brain homogenate of transgenic mice not injected with synthetic prions. Finally, the clinical and histopathological presentation of the disease was different from the usual disease in mice. The authors argued that this result may be due to the creation of a new strain of prions.

A strategy that is regarded as more promising is the generation of infectivity by in vitro conversion of PrP^{C} because in these experiments the transformation process is triggered, catalyzed, and templated by brain-derived PrP^{res}. The cell-free conversion system developed by Caughey and coworkers (Kocisko et al., 1994) uses purified PrP^{C} mixed with stoichiometric amounts of purified PrP^{res}. The low yield of PrP^{res} formation resulting from this system and the nonphysiological conditions used had made it difficult to evaluate the biological and structural properties of the newly converted protein. However, Collinge and coworkers took advantage of the species-barrier phenomenon to test infectivity of newly generated PrP^{res} under conditions in which the PrP^{res} from the inoculum would not be infectious (Hill et al., 1999). The results of these experiments argued that cell-free-generated PrP^{res} was not infectious. A recent study attempting to analyze infectivity of PMCA-generated PrP^{res} produced inconclusive results (Bieschke et al., 2004). The most likely reason for this was that the level of PrP^{res} produced in vitro was still not high enough to have a clear separation from the infectivity of the material used to generate the misfolded protein. In addition, an important control experiment analyzing the parallel effect of the sonication procedure on brain-derived infectivity was missing. We have obtained similar results in other infectivity studies in which animals inoculated with PrP^{res} obtained after a low level (10- to 50-fold) of amplification led to inconclusive results regarding the infectivity of newly generated protein (data not shown). However, in experiments in which a higher level of amplification (>200-fold) was obtained in a single round of PMCA, a significant increase in infectivity was observed (data not shown).

In this study we have further optimized the PMCA procedure to reach very high levels of PrP^{res} conversion. Furthermore, we have been able to serially amplify PrP^{res} in vitro indefinitely. As a result of many rounds of PMCA following serial dilution of brain infectious material, we have been able to generate large quantities of newly synthesized PrP^{res} in the absence of any molecules of the brain-derived misfolded protein. This in vitro-generated PrP^{res} isoform has identical biochemical and structural properties to the protein used to begin the conversion, and, more importantly, the in vitro-generated PrP^{res} is clearly associated with infectivity. Our results represent the first time in which prion infectious material has been generated in vitro to produce an in vivo disease in wild-type animals with characteristics identical to the disease produced by brain-isolated prions. These results provide some of the strongest evidence in support of the prion hypothesis. The fact that a given quantity of in vitro-generated PrP^{res} is associated with a lower degree of infectivity as compared with brain-derived infectious material may explain the inconclusive results from Bieschke and coworkers (Bieschke et al., 2004) and our own previous data using a low level of amplified material for inoculation. The reason for the lower level of infectivity in PMCA-produced PrP^{res} is unknown and currently under investigation, but we can envision at least three possibilities. First, although the samples contained the same amount of PrP^{res} as analyzed by Western blot after SDS-PAGE, the protein molecules might have been organized into a different number of infectious units. For example, a large production of PrP^{res} in vitro may have led to the formation of big aggregates that might be less efficient for propagating infectivity in vivo, or sonication may have produced too small aggregates that were not very efficient in maintaining infectivity. Second, it is possible that in vitro-generated and in vivo-derived infectious agents may contain different proportions of resistant and sensitive forms of PrP. In our biochemical quantitation we only take PrP^{res} into account. Third, we may have generated a different prion strain after repeated in vitro amplification reactions. Although the biochemical, histological, and clinical characteristics of the disease produced by in vitro-generated PrP^{res} were identical to the disease originating from brain-derived infectious material, the extended incubation periods, both in the first and second passages, support a possible new strain. Additional experiments of serial transmission and titration are needed to reach definitive conclusions.

A central facet of the prion hypothesis is that prion replication is a cyclical process and that newly produced PrP^{res} can further propagate protein misfolding; in this way, prions can continue replicating across animals and generations. Our results show that this process of autocatalytic generation of PrP^{res} can be mimicked in vitro by PMCA, indicating that prions can be "cultured" in vitro indefinitely, thus enabling the detailed study of their properties. In vitro-produced PrP^{res} exhibited properties strikingly similar to those of the protein isolated from scrapie-affected animals, including secondary structure, Western blot profile, protease resistance, detergent insolubility, resistance to denaturation by heat and chaotropic agents, aggregation into rod-like structures, efficiency in inducing the misfolding of the normal PrP^{C}, and in vivo infectivity. These findings indicate that PMCA is a valuable tool for studying the molecular basis of prion conversion and the factors involved in this process as well as for identifying novel compounds that inhibit this pathological event. PMCA might also be useful for studying the mechanism of the species barrier and assessing whether the nature of the prion strains is indeed encoded on the structure of the misfolded protein. Finally, the high levels of amplification resulting from our improved and automated PMCA procedure may have important applications for TSE di-

agnosis by allowing the highly sensitive detection of PrP^{res}.

Experimental Procedures

Preparation of Tissue Homogenates

Healthy and sick animals were perfused with phosphate-buffered saline (PBS) plus 5 mM EDTA previous to harvesting the tissue. Ten percent brain homogenates (w/v) were prepared in conversion buffer (PBS containing 150 mM NaCl, 1.0% Triton X-100, 4 mM EDTA, and the complete™ cocktail of protease inhibitors [Boehringer Mannheim, Mannheim, Germany]). The samples were clarified by a brief low-speed centrifugation (1500 rpm for 30 s) using an Eppendorf (Hamburg, Germany) centrifuge, model 5414.

PrP^{res} Purification

One gram of brain tissue was homogenized in 5 ml of cold PBS containing protease inhibitors. For PMCA-generated PrP^{res}, the total sample was processed after the last amplification in the same way as brain homogenate. The samples were mixed with 1 volume of 20% sarkosyl, and the mixture was homogenized and sonicated until a clear preparation was obtained. Samples were centrifuged at 5000 rpm for 15 min at 4°C. The pellet was discarded, the supernatant was mixed with 1/3 volume of PBS containing 0.1% SB-314, and samples were centrifuged in a Biosafe Optima MAX ultracentrifuge (Beckman Coulter, Fullerton, California) at 100,000 × g for 3 hr at 4°C. The supernatant was discarded and the pellet was resuspended in 600 μl of PBS containing 0.1% SB-314 and 10% NaCl and sonicated. The resuspended pellet was layered over 600 μl of PBS containing 20% saccharose, 10% NaCl, and 0.1% SB-314 and centrifuged for 3 hr at 4°C. The supernatant was discarded and the pellet was resuspended in 300 μl of PBS containing 0.1% SB-314 and sonicated again. After sonication the samples were incubated with PK (100 μg/ml) for 2 hr at 37°C and shaken. The digested sample was layered over 100 μl of PBS containing 20% sarkosyl, 0.1% SB-314, and 10% NaCl and centrifuged for 1 hr 30 min at 100,000 × g. The final pellet was resuspended in 100 μl of PBS and sonicated. The sample was stored at −80°C. Purity was analyzed by silver staining and amino acid composition analysis.

PMCA Procedure

Although the principle of PMCA remains the same as in our original publication (Saborio et al., 2001), the system has been optimized and automated, thus enabling the routine processing of many more samples at the same time and reaching a higher conversion efficiency. The detailed protocol, including troubleshooting, has been recently published elsewhere (Castilla et al., 2004b; Saá et al., 2004). Aliquots of normal and scrapie brain homogenate prepared in conversion buffer were mixed and loaded onto 0.2 ml PCR tubes. Tubes were positioned on an adaptor placed on the plate holder of a microsonicator (Misonix Model 3000, Farmingdale, New York) and programmed to perform cycles of 30 min incubation at 37°C followed by a 40 s pulse of sonication set at 60% potency. Samples were incubated, without shaking, immersed in the water of the sonicator bath.

Protease-Resistance Assay

The profile of PK sensitivity for in vitro- and in vivo-generated PrP^{res} was studied by subjecting samples to incubation for 60 min at 45°C with different concentrations of PK ranging from 0 to 10,000 μg/ml. The digestion was stopped by adding electrophoresis sample buffer. For all other experiments involving PK digestion, the standard conditions used consisted of incubating the samples with 50 μg/ml of the enzyme for 60 min at 45°C.

PrP^{res} Quantification

PrP^{res} concentration was estimated by Western blotting followed by densitometric analysis, using known concentrations of hamster recombinant PrP^{C} (Prionics Inc, Zurich, Switzerland). To obtain a reliable and robust quantification, we ran several different dilutions of the sample in the same gel as the standard, thus avoiding artifacts due to saturation of the signal or to too weak a signal. In some selected samples, quantification was confirmed by single ELISA technique after PK digestion, using plates coated with 3F4 antibody.

Detergent Solubility Assay

Samples containing brain homogenate and in vitro- or in vivo-generated PrP^{res} were incubated in the presence of 10% sarkosyl for 30 min at 4°C. Thereafter, samples were centrifuged at 100,000 × g for 1 hr in a Biosafe Optima MAX ultracentrifuge (Beckman Coulter), and the pellet of the centrifugation was then resuspended in conversion buffer plus electrophoresis sample buffer. Equivalent aliquots of supernatant and pellet were analyzed using immunoblotting. In some experiments, prior to the addition of sarkosyl, samples were incubated with different concentrations of guanidine hydrochloride for 2 hr at room temperature and shaken. Thereafter, sarkosyl was added and the soluble and insoluble proteins were separated using centrifugation.

PrP^{res} Detection

Proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, electroblotted into nitrocellulose membrane, and probed with 3F4 antibody (Kascsak et al., 1987) (dilution 1:5000). The immunoreactive bands were visualized by enhanced chemiluminescence assay (Amersham, Piscataway, New Jersey).

Protein Deglycosylation Assay

PrP^{res} samples were first digested with PK as described above. After addition of 10% sarkosyl (final concentration), samples were centrifuged at 100,000 × g for 1 hr at 4°C, the supernatant was discarded, and the pellet was resuspended in 100 μl of glycoprotein denaturing buffer (New England Biolabs, Beverly, Massachusetts) and incubated for 10 min at 100°C. Thereafter, 26 μl of 50 mM sodium phosphate (pH 7.5) containing 1% nonidet P-40 and 3 μl of peptide N-glycosidase F (New England Biolabs) was added. Samples were incubated for 2 hr at 37°C, the reaction was stopped by adding electrophoresis buffer, and samples were analyzed by Western blot.

Fourier Transform Infrared Spectroscopy

Solutions or suspensions containing highly purified PrP^{res} (5 mg/ml) were prepared in PBS. Two hundred microliters was loaded into an infrared cell, and samples were dried for 30 min under a nitrogen flow. Spectra were recorded with a Bruker (Billerica, Massachusetts) model 66v/S FTIR spectrophotometer at 25°C. For each spectrum, 512 scans were collected with a 2 cm^{-1} resolution and a 1 cm^{-1} interval from 4000–700 cm^{-1}. Smoothing and Fourier self-deconvolution were applied to increase the spectral resolution in the amide I region (1700–1600 cm^{-1}), and iterative fitting to lorentzian line shapes was carried out using the Grams software from Thermo Corporation (San Jose, California) to estimate the proportion of each secondary-structure element.

Electron Microscope

Samples of PrP^{res} either highly purified from the brain or generated by PMCA amplification were resuspended in PBS, placed onto carbon formvar-coated 300-mesh nickel grids, and stained for 60 s with 2% uranyl acetate. Grids were analyzed using a Philips EM 410 (FEI Company, Hillsboro, Oregon) electron microscope at 60 kV.

PrP^{res} Thermal Inactivation

Equivalent samples of brain homogenates containing in vivo-derived and PMCA-generated PrP^{res} were incubated for 20 min at different temperatures (room temperature, 100°C, 110°C, 120°C, and 140°C). Thereafter, aliquots were taken, diluted into normal brain homogenate, and subjected to PMCA amplification. Formation of new PrP^{res} was monitored using Western blot after PK digestion.

In Vivo Infectivity Studies

Syrian golden hamsters were used. Animals were 4 to 6 weeks old at the time of inoculation. Anesthesized animals were injected stereotaxically in the right hippocampus with 1 μl of the sample using a computerized perfusion machine that delivered the sample into

the brain at a rate of 0.1 μl/min. For the second-passage experiments, animals were inoculated i.p. with 100 μl of the material. The onset of clinical disease was measured by scoring the animals twice per week using the following scale: 1, normal animal; 2, mild behavioral abnormalities, including hyperactivity and hypersensitivity to noise; 3, moderate behavioral problems, including tremor of the head, ataxia, wobbling gait, head bobbing, irritability, and aggressiveness; 4, severe behavioral abnormalities, including all of the above plus jerks of the head and body and spontaneous backrolls; 5, terminal stage of the disease, in which the animal lies in the cage and is no longer able to stand up. Animals scoring level 4 for two consecutive weeks were considered sick and were sacrificed to avoid excessive pain using exposure to carbonic dioxide. Brains and other tissues were extracted and analyzed histologically. The right cerebral hemisphere was frozen and stored at −70°C for biochemical examination of PrP^{res} using Western blot analysis; the left hemisphere was fixed in 10% formaldehyde solution, cut into sections, and embedded in paraffin. Serial sections (6 μm thick) from each block were stained with hematoxylin and eosin, using standard protocols or incubated with monoclonal antibodies recognizing PrP or the glial fibrillary acidic protein. Immunoreactions were developed using the peroxidase-antiperoxidase method, following manufacturer specifications. Antibody specificity was verified by absorption.

Supplemental Data

Supplemental Data include three figures and one reference and are available with this article online at http://www.cell.com/cgi/content/full/121/2/195/DC1/.

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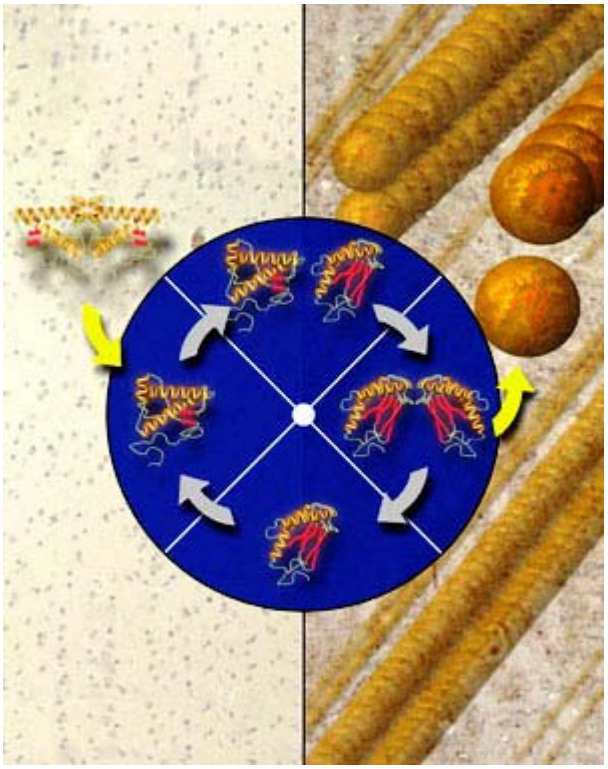
21 April 2005

Proof Positive for Prions

A new study has taken another step toward proving the "**prion** hypothesis"—the idea that rogue proteins are responsible for a host of fatal neurological diseases, including Mad Cow Disease and scrapie.



Prion factory. A schematic representation of the cyclic process by which a molecule of PrP^{C} (*left side of circle*) interacts with and is converted into PrP^{Sc} (*right side of circle*). PrP^{Sc} damages normal brain tissue (*left panel*) by forming long fibers (*right panel*).
CREDIT: Eric Smith, Harvard University

According to the **prion** hypothesis, the disease-causing protein, PrP^{Sc}, makes copies of itself inside the brain, multiplying until it forms fibers that destroy neurons and eventually kill the animal. PrP^{Sc} has the same amino acid sequence as a normal brain protein called PrP^{C}, but folds into a different three-dimensional shape. It replicates by corrupting any PrP^{C} it encounters, twisting it into a new PrP^{Sc}, which in turn transforms more PrP^{C}. Although recent research has overwhelmingly supported the **prion** hypothesis [ScienceNOW 15 October, 2003], one vital experiment was missing: No one had induced a **prion** disease in a healthy animal using pure PrP^{Sc} made in a test tube.

In work reported in the 22 April *Cell*, neurobiologist Claudio Soto of the University of Texas Medical Branch in Galveston and colleagues come close to achieving that goal. To generate high levels of PrP^{Sc}, Soto's group performed 20 rounds of "infection" in a test tube. In the first round, the group mixed ground up healthy brain with brain material from a scrapie-infected animal. Just as in an

intact animal, the PrP^{C} in the healthy brain material was converted to PrP^{Sc}. In subsequent rounds, they used material from the previous round to infect fresh healthy brain material. The procedure yielded billions of molecules of PrP^{Sc} from just a few starting molecules. Although some of the original scrapie brain material was carried through the early rounds of the experiment, it eventually became diluted to the point where it disappeared from the sample. Nonetheless, the material was very infectious, efficiently causing scrapie when injected into the brains of hamsters.

The caveat, UCSF neurobiologist Giuseppe Legname notes, is that because the researchers started with scrapie brain, not pure PrP^{Sc}, something else that contributes to scrapie may have been amplified along with PrP^{Sc}. However, Legname says, this system could be used to detect the low levels of **prion**s present in the blood of infected animals. Such a test is "desperately needed," he says, to reduce the chances that **prion**s will be spread through food or medical blood products.

--KAREN ROSS

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Plant biology

Chestnuts clock out in winter

Proc. Natl Acad. Sci. USA
doi:10.1073/pnas.0408549102 (2005)

Do plants put their internal clocks on pause when winter arrives? According to Alberto Ramos *et al.*, such a disruption indeed takes place in the European chestnut.

Ramos *et al.* examined parts of adult trees — including stems, leaves and buds — for gene products associated with the circadian clock. They found that the chestnut clock behaves like that of the flowering plant *Arabidopsis thaliana* during the growing season, but acts differently in colder months.

During June, expression of the genes *CsTOC1* and *CsLHY* (counterparts of two *Arabidopsis* clock genes) followed strong cycles. But their expression remained high and did not oscillate when the trees encountered colder temperatures and entered dormancy. Normal cycling of *CsTOC1* and *CsLHY* expression resumed when chestnut seedlings in the lab were exposed to warmer temperatures. The findings suggest that woody and herbaceous plants use different methods for adjusting their clocks to the cold. **Roxanne Khamsi**

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Network theory

On Broadway

Science **308**, 697–702 (2005)

Another opening, another show. But what makes it a success? According to Roger Guimerà and colleagues' analysis of Broadway musicals, the answer is maximizing experience and innovation by means of a well-mixed and well-connected production team, with a dash of fresh faces.

Underlying such teams is a network of collaborations, and Guimerà *et al.* investigate these in terms of team size, which grows with the complexity of the task; the probability of being an established team member, or 'incumbent', hence experienced and well connected; and the likelihood that an incumbent chooses to work again with a previous collaborator.

Simulating team-building in the phase space determined by these parameters, the authors find a phase transition to a large connected cluster. This means that there are optimum values of the parameters for which a complex network of contacts exists that is the basis for success.

The same is true of some research teams. For collaborations in social psychology, economics and ecology — and using journal impact factor as a measure of success — Guimerà *et al.* conclude that it's getting the right combination of newcomers and incumbents that counts. Curiously, however, astronomy doesn't fit the pattern. **Alison Wright**

Cell biology

Prion progress

Cell **121,** 195–206 (2005)

Are prions — infectious proteins — the sole cause of disorders such as Creutzfeldt–Jakob disease and scrapie? Joaquín Castilla *et al.* add to the case that they are.

According to the prion hypothesis, normal prion proteins become twisted out of shape, and then convert further normal proteins into the same warped form. One way of testing the hypothesis is to generate misshapen prions *in vitro* and see if they cause disease *in vivo*. Researchers have succeeded in doing this with yeast prions. They have also generated mammalian prion-like protein fragments *in vitro*, and used them to cause disease in transgenic mice engineered to overexpress the same fragments.

Castilla *et al.* go further. Tweaking one of their own techniques, called 'protein misfolding cyclic amplification', they used extracts of scrapie-infected hamster brains to 'seed' the conversion of normal prion proteins into the abnormal form *in vitro*. Numerous cycles produced samples containing practically none of the original brain extracts. When wild-type hamsters were infected with prions that had been generated *in vitro*, the animals showed typical signs of scrapie. **Amanda Tromans**

Chemistry

Molecular bends

Angew. Chem. Int. Edn Engl. **44,** 2382–2385 (2005)

Heating usually speeds up chemical reactions — the extra energy causes molecules to vibrate more, which stretches interatomic bonds and makes them more likely to break. Hans A. Bechtel and colleagues find that heating also increases the bending of molecular bonds.

Compared with stretching vibrations, bending vibrations have a lower frequency and are less energetic, so have remained largely unexplored. Bechtel *et al.* studied the reaction of methane (CH_4) with atomic chlorine, which occurs in atmospheric chemistry and is a common model for investigating reaction rates.

They show that, counterintuitively, bending excitation increases the chances of methane reacting by at least a factor of two, implying that shearing motion, as well as stretching, can help to break C–H bonds. They also find that the energy involved in the movement of methane's atoms leads almost exclusively to motion of the escaping reaction products, rather than being retained as vibrational energy in the products — something not predicted by theory. Targeting bending excitation of molecules may boost rates in other reactions, the authors suggest. **Mark Peplow**

Drug delivery

Shake-up for injections

Colloids Surf. A **260,** 7–16 (2005)

Many drugs are built on a hydrocarbon molecular scaffolding, and so are hydrophobic and rather insoluble in water. This makes intravenous injection of water-based solutions impossible. Often, such drugs are dissolved in oils that are then dispersed in water using surfactant emulsifiers — but both the oils and the surfactants may degrade into harmful compounds.

R. M. Pashley and co-workers recently found that water and oil mixtures from which dissolved air has been removed are much better at dispersing oily and greasy substances. Pashley and M. J. Francis have now shown that degassed oils dispersed in degassed water could be a suitable method for delivering hydrophobic drugs.

Simply shaking common drug-delivery oils, in degassed form, such as soybean oil, degassed water for a few seconds produces a dispersion of uniform droplets about 1–3 μm in diameter, which could act as robust 'parcels' for the safe intravenous injection of drugs. Liquid, water-insoluble drugs such as propofol (a sedative) and griseofulvin (used to treat skin infections) could be dispersed directly in degassed water without any carrier oil. **Philip Ball**