Cell-free propagation of prion strains

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Prions are the infectious agents responsible for prion diseases, which appear to be composed exclusively by the misfolded prion protein (PrP^{Sc}). Disease is transmitted by the autocatalytic propagation of PrP^{Sc} misfolding at the expense of the normal prion protein. The biggest challenge of the prion hypothesis has been to explain the molecular mechanism by which prions can exist as different strains, producing diseases with distinguishable characteristics. Here, we show that PrP^{Sc} generated *in vitro* by protein misfolding cyclic amplification from five different mouse prion strains maintains the strain-specific properties. Inoculation of wild-type mice with *in vitro*-generated PrP^{Sc} caused a disease with indistinguishable incubation times as well as neuropathological and biochemical characteristics as the parental strains. Biochemical features were also maintained upon replication of four human prion strains. These results provide additional support for the prion hypothesis and indicate that strain characteristics can be faithfully propagated in the absence of living cells, suggesting that strain variation is dependent on PrP^{Sc} properties.

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Introduction

Prions are the unconventional infectious agents responsible for prion diseases, a group of fatal, neurodegenerative disorders, including Creutzfeldt–Jakob disease (CJD) in humans, scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle and chronic wasting disease in deer, among others (Collinge, 2001). The infectious agent appears to be composed exclusively by a misfolded version of the prion protein (PrP^{Sc}) that replicates in the body in the absence of

nucleic acids by inducing the misfolding of the cellular prion protein (PrP^C) (Prusiner, 1998). Although recent studies showing *in vitro* generation of infectious material by inducing or amplifying PrP misfolding have provided strong support for the prion hypothesis (Legname *et al*, 2004; Castilla *et al*, 2005; Deleault *et al*, 2007), it remains still highly controversial (Somerville, 2000; Chesebro, 2003; Soto and Castilla, 2004; Weissmann, 2004).

One of the main difficulties of the prion hypothesis has been to provide a molecular explanation for the prion strain phenomenon (Chesebro, 1998; Somerville, 2002; Aguzzi *et al*, 2007; Morales *et al*, 2007). Most TSEs are known to exhibit various ‘strains’ characterized by differences in incubation periods, clinical symptoms and biochemical and neuropathological features (Bruce, 2003; Aguzzi *et al*, 2007; Morales *et al*, 2007). In infectious diseases associated with conventional microbial agents (virus, bacteria and so on), different strains arise from mutations or polymorphisms in the genetic make-up of the agent. To reconcile the infectious agent composed exclusively of a protein with the strain phenomenon, it has been proposed that the strain characteristics are dependent on slightly different conformation or aggregation states of PrP^{Sc}, which can faithfully replicate at expenses of the host PrP^C (Telling *et al*, 1996; Prusiner, 1998; Morales *et al*, 2007). These different folding states of PrP^{Sc} may lead to selectively targeting distinct brain regions producing the diverse neuropathological alterations and clinical symptoms characteristic of prion strains. Support for this concept came from various studies showing that PrP^{Sc} from different strains have noticeable distinct biochemical properties and secondary structures (Caughey *et al*, 1998; Safar *et al*, 1998). However, up to now it is not known whether such differences are the cause or simply another manifestation of the prion strain phenomenon. Furthermore, the concept that a single protein can provide the conformational flexibility and the mechanism to propagate diverse strains is very intriguing and unprecedented. The definitive proof that the strain phenomenon is encoded in the PrP^{Sc} structure would be to reproduce in the test tube the folding of PrP^{Sc} associated with different strains and to show that the *in vitro*-generated infectious proteins maintain the *in vivo* strain characteristics. Important findings in this direction have been obtained for yeast prions, which are a group of ‘infectious proteins’ that behave as non-Mendelian genetic elements and transmit biological information in the absence of nucleic acid (Wickner *et al*, 1995). Compelling evidences have provided support for the prion nature of several yeast proteins, including Sup35p, Ure2p, Rnq1 (Uptain and Lindquist, 2002; Chien *et al*, 2004; Wickner *et al*, 2004). Recent studies showed that bacterially produced N-terminal fragments of Sup35p and Ure2p when transformed into amyloid fibrils were able to propagate the prion phenotype to yeast cells (King and Diaz-Avalos, 2004; Tanaka *et al*, 2004; Brachmann *et al*, 2005). Remarkably, infection of yeasts with recombinant Sup35 folded in different conformations led to

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the generation of distinct prion strains *in vivo* (Tanaka *et al*, 2004; Brachmann *et al*, 2005), indicating that structural differences in the infectious protein determine prion strain variation.

On the basis of theoretical considerations about the mechanisms of prion conversion, we have developed a strategy to reproduce PrP^{Sc} replication *in vitro* with a similar efficiency to the *in vivo* process, but with accelerated kinetics (Saborio *et al*, 2001). This system called PMCA (protein misfolding cyclic amplification) was designed to mimic PrP^{Sc} autocatalytic replication. In a cyclic manner, conceptually analogous to PCR cycling, a minute quantity of PrP^{Sc} (as little as one single particle) is incubated with excess PrP^C to enlarge the PrP^{Sc} aggregates that are then sonicated to generate multiple smaller units for the continued formation of new PrP^{Sc} (Saborio *et al*, 2001; Saá *et al*, 2006). PMCA confirms a central facet of the prion hypothesis, which is that prion replication is an autocatalytic process and that newly produced PrP^{Sc} can further propagate the protein misfolding (Soto *et al*, 2002). We have previously reported proof-of-concept experiments in which the technology was applied to replicate the misfolded protein from diverse species (Soto *et al*, 2005). The newly generated protein exhibits the same biochemical, biological and structural properties as brain-derived PrP^{Sc} and is infectious to wild-type animals, producing a disease with similar characteristics as the illness produced by brain-isolated prions (Castilla *et al*, 2005).

The main goal of this study was to assess whether the specific biological, biochemical and infectious properties of distinct prion strains can be maintained after *in vitro* replication. For this purpose, we propagated five different mouse and four distinct human prion strains *in vitro* by PMCA using as substrate PrP^C from the same normal mouse or human transgenic mouse brain extract, respectively. PrP^{Sc} associated with each strain was serially diluted and replicated *in vitro* to produce misfolded protein free from brain PrP^{Sc} inoculum. *In vitro*-generated PrP^{Sc} maintains the strain-specific biochemical properties and more importantly upon injection into wild-type mice produced a disease with indistinguishable characteristics as the parental strain. These results suggest that all strain-associated features can be maintained by cell-free formed PrP^{Sc}, suggesting that the prion strain phenomenon is enciphered on the characteristics of the misfolded prion protein.

Results

In vitro propagation of prion strains

To analyse whether *in vitro* replication of prion strains faithfully propagates the biochemical properties of PrP^{Sc}, we used five well-characterized strains from mouse and four from human. As many as 20 different prion strains have been identified in mouse after inoculation of animals with prions from diverse origins (Fraser and Dickinson, 1973; Kimberlin, 1976; Bruce, 2003). Four of the mouse strains used in this study (RML, 139A, ME7 and 79A) are originated from different scrapie sources and have been adapted into mouse by repetitive passages. These mouse-adapted scrapie strains have been shown to have some differences in brain lesion profiles, incubation times in diverse mouse backgrounds and susceptibility to PrP polymorphisms (Fraser and Dickinson, 1973; Bruce *et al*, 1991). On the other hand, 301C is a strain

originated from cattle BSE, which has been serially passed in wild-type mice (Bruce *et al*, 2002). Mouse brain homogenates from animals experimentally infected with these different strains were diluted 10-fold into 10% normal mice brain homogenate. Samples were either immediately frozen or subjected to 96 PMCA cycles. The amplified samples were further diluted 10-fold and a new round of 96 PMCA cycles was carried out. This procedure of serial dilution/amplification was repeated many times to reach a 10⁻²⁰ total dilution of the brain infectious material and these samples were used in the studies described below. In our estimation, this degree of dilution is at least 1 million-fold higher than what is necessary to remove the last molecule of PrP^{Sc} coming from the brain inoculum and at least a trillion (10¹²)-fold more than the last infectious dilution (Castilla *et al*, 2005). As determined by western blot after proteinase K (PK) digestion, a protease-resistant product was observed in all these amplifications, which remained constant despite the dilutions (data not shown). These results suggest that PrP^{Sc} is being produced *in vitro* and the newly generated PrP^{Sc} is capable of sustaining replication, as demonstrated before for hamster prions (Castilla *et al*, 2005). We conclude from these results that PMCA enables an infinite replication of PrP^{Sc} *in vitro* and the large dilution performed (10⁻²⁰) guarantees that the sample contains exclusively *in vitro*-produced misfolded protein. Assessment of the western blot profile after PK treatment for PMCA-generated PrP^{Sc} compared with *in vivo*-produced misfolded protein from each strain shows that both the proportion of glycoforms and the electrophoretic mobility were conserved after *in vitro* propagation (Figure 1). As shown in Figure 1A, the scrapie-derived mouse strains (RML, ME7, 139A and 79A) have a glycoform distribution dominated by the mono-glycosylated form, whereas in 301C PrP^{Sc} the most abundant isoform is the di-glycosylated species. Importantly, the glycoform ratio in PMCA-generated PrP^{Sc} was very similar to the brain-derived protein. The relative migration of the protein in gel electrophoresis was also strikingly similar, which is easily observed upon deglycosylation when comparing only the size of the non-glycosylated protein (Figure 1B). PrP^{Sc} from scrapie-adapted mouse strains migrate with an estimated molecular weight of 21 kDa, whereas the bovine-derived 301C protein exhibits a ~19 kDa size. These findings indicate that the PK cleavage site in PrP^{Sc} generated *in vitro* by PMCA is similar to the site in which the protein obtained from the brain of sick animals is cleaved by this enzyme. This is important because differences in proteolytic cleavage likely reflect distinct foldings of the protein in diverse strains (Marsh and Bessen, 1994; Collinge *et al*, 1996; Chen *et al*, 2000; Gretzschel *et al*, 2005). It is noteworthy that the PrP^C substrate used for conversion was the same for all strains and thus the molecular characteristics of PrP^{Sc} in the inoculum are responsible for the propagation of the different biochemical properties of the protein. A control experiment in which samples of brain homogenate from 10 different healthy mice were serially diluted into itself and subjected to the same number of PMCA cycles as described above, but in the absence of PrP^{Sc} inoculum, did not show any protease-resistant PrP under the conditions used (data not shown). Although we have been able to generate infectious PrP^{Sc} 'de novo' (without the addition of brain PrP^{Sc}) recently, this requires modification of some PMCA parameters (paper under review) and

Figure 1 Biochemical properties of *in vitro*-generated PrP^{Sc} from different mouse and human strains. Brain homogenates from sick individuals (mice or humans) were diluted 10-fold into normal brain homogenate and subjected to 96 cycles of PMCA, as described in Materials and methods. 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 the inoculum. (A) Aliquots containing similar amounts of brain-derived or PMCA-generated (after a 10⁻²⁰ dilution) PrP^{Sc} from different mouse strains were subjected to proteinase K (PK) digestion (50 µg/ml for 60 min at 37°C) and loaded onto SDS-PAGE. Immunoreactive bands were observed using western blot. For clarity, the figure was composed from blots with different levels of exposure to enable a direct comparison of the position of the bands. (B) Aliquots of both proteins were subjected to deglycosylation by treatment with peptide N-glycosidase F for 2 h at 37°C. (C) Electrophoretic pattern of four different types of *in vitro*-generated or brain-derived human PrP^{Sc}. (D) The size of the PK-resistant PrP^{Sc} fragment in diverse human strains was assessed by western blot after deglycosylation. All samples were digested with PK before western blot, except in the normal brain homogenate (NBH), used as a control of PrP^C migration. B: brain-derived samples; P: PMCA-generated samples.

under standard PMCA conditions, as those used in this study, spontaneous generation of infectious material does not occur. These findings suggest that *de novo* formation of PrP^{Sc} can be experimentally distinguished from replication of pre-formed PrP^{Sc}, indicating that the biochemical, conformational or stability properties of the PrP structures involved in both processes are probably different.

To further assess whether the PrP^{Sc} features are maintained after *in vitro* replication by PMCA, we tested four different human strains with well-established differences in the biochemical properties of PrP^{Sc} (Gambetti *et al*, 2003). The samples came from patients affected by vCJD, and three distinct types of sCJD (types MM1, MM2 and VV2). PrP^{Sc} from vCJD and sCJD types MM1 and MM2 was amplified using as substrate transgenic mice brain expressing human PrP with MM genotype at position 129 and the sCJD type VV2 was amplified using transgenic mice expressing VV PrP^C. Little or no amplification was obtained when PrP^{Sc} and PrP^C have different polymorphism at position 129. Samples were subjected to the same scheme of serial PMCA and dilutions as described in the mouse experiment above to dilute out the PrP^{Sc} inocula used to trigger the reaction. Comparison of the glycoform proportion (Figure 1C) and the electrophoretic mobility after PK and deglycosylation (Figure 1D) between brain-derived and PMCA-generated PrP^{Sc} for each strains clearly show that the biochemical features were maintained upon *in vitro* propagation. Unseeded PMCA reactions of healthy human PrP transgenic mice brain homogenates did not show '*de novo*' PrP^{Sc} generation under the conditions tested (data not shown).

***In vitro*-generated PrP^{Sc} maintains strain-specific infectivity properties**

To determine the infectious capability of *in vitro*-generated PrP^{Sc} and to assess the preservation of strain characteristics,

Table 1 Infectivity of *in vitro*-generated prions

Prion strain	Brain	PMCA	PMCA—second passage	P-value*
RML	159 ± 7	164 ± 12	148 ± 1	0.382
ME7	156 ± 2	163 ± 6	164 ± 2	0.249
301C	189 ± 2	181 ± 2	183 ± 4	0.145
139A	162 ± 1	167 ± 6	169 ± 1	0.338
79A	161 ± 3	154 ± 4	154 ± 3	0.253
None	> 500	> 500	ND	—

Groups of five wild-type mice were injected i.c. with similar quantities of protease-resistant PrP^{Sc} either derived from the brain of sick animals (brain) or generated by PMCA *in vitro* (PMCA). Also shown is the second passage of the PMCA group. The values represent the average ± standard error of the time in which animals were killed with definitive signs of disease. The attack rate in all cases was 100%, except in the control experiment in which the normal brain homogenate was subjected to PMCA in the absence of any infectious material. ND: not done.

*The P-value was calculated by one-way ANOVA using the software GraphPad Instat, version 3.05.

we inoculated the five mouse strains into wild-type animals. Groups of mice were injected intracerebrally (i.c.) with a similar quantity of either brain-derived or PMCA-produced PK-resistant PrP^{Sc}. Appearance of clinical signs was monitored over time as described in Materials and methods. The first alterations observed were rough coat initially in the neck, which then extended to the lower back. This was followed by hunchback, urogenital lesions, cachexia and ataxia. Unfortunately, no substantial differences in clinical signs are evident among these strains other than the observation that 301C has a more severe urogenital damage, including abundant pus secretion. Nevertheless, the time of appearance of clinical alterations (Table 1), and the type and severity of the signs were very similar in the animals inoculated with infectious material obtained from sick brain

or produced *in vitro* by PMCA. None of the animals inoculated with normal brain homogenate subjected or not to the same regimen of serial PMCA amplification showed disease signs up to 500 days post-inoculation (Table I). Interestingly, in none of these experiments we found a significant delay on the incubation time for the *in vitro*-generated prions compared with the *in vivo*-produced infectious agent, as we reported earlier in our experiments with hamster 263K scrapie prions (Castilla *et al*, 2005). These findings indicate that the infectivity associated with PMCA-generated PrP^{Sc} was the same as for misfolded protein obtained from the brain in all the mouse strains studied. A second passage of the *in vitro*-generated infectious material showed the same incubation periods, suggesting that the infectious agent was stable (Table I). Similar results were obtained when the material was inoculated intraperitoneally, albeit with longer incubation times (Data not shown). Statistical analysis of the incubation periods showed that the differences between the brain-derived material or PMCA-generated infectivity (first and second passages) were not significant for any of the strains studied (see *P*-values in Table I). Conversely, the incubation times for 301C were highly significantly different from all the other strains, as assessed by two-way ANOVA ($P < 0.001$). The differences between the ovine-derived strains are nonsignificant, except for 139A versus 79A ($P = 0.0031$).

Besides incubation time, one of the main differences among prion strains is the distribution and severity of damage in distinct regions of the brain (Fraser and Dickinson, 1973). To assess whether *in vitro*-generated infectious agent maintains the neuropathological signature of the parental strain, we evaluated the degree of vacuolation in various brain areas of infected animals, as described in Materials and methods. The profile of spongiform degeneration obtained for each strain using brain-derived or PMCA-generated material was statistically undistinguishable (Figures 2 and 3), indicating again that *in vitro* propagation of prions maintains the strains properties. As shown in Figure 3, statistical analyses of the brain lesion profile enable to differentiate three groups of strains: 301C, 79A and the other three ovine-derived strains (RML, 139A, ME7), which are not significantly different in their pattern of vacuolation. 301C-infected mice exhibit large extent of spongiosis in medulla and hippocampus, medium degree of damage in colliculus, hypothalamus and thalamus and very little vacuolation in cerebellum and cortex (Figures 2 and 3). On the other hand, the ovine strains RML, 139A and ME7 have a large extent of spongiform degeneration in cerebellum and only little damage in colliculus and hypothalamus. Finally, the 79A strain produces a more even pattern of vacuolation in the eight brain areas studied. These neuropathological differ-

Figure 2 Histopathological profile of spongiform brain degeneration in mice inoculated with *in vivo*- and *in vitro*-produced prion strains. Pictures showing the vacuolation extent in eight brain regions in sick mice inoculated with brain-derived (brain) or PMCA-generated (PMCA) prions of three of the strains used in this study (ME7, 79A and 301C). As a control, we showed the brain staining of animals inoculated with brain homogenate subjected to the same serial PMCA procedure. These animals did not show clinical signs of the disease and were killed 500 days after inoculation. Samples were processed and stained by haematoxylin-eosin as described in Materials and methods and visualized at $\times 100$ magnification.

Figure 3 Profile of vacuolation in different brain areas of mice inoculated with different strains of *in vivo*- and *in vitro*-generated prions. Brains from mice clinically sick by inoculation with either brain-derived or PMCA-generated prions from the five strains used in this study were analysed histologically for spongiform degeneration after haematoxylin-eosin staining, as described in Materials and methods. Eight different brain areas were selected and the values represent the average $\pm$ s.e. of the extent of vacuolation from the five animals analysed. The brain areas studied were the following: (1) dorsal medulla; (2) cerebellar grey matter; (3) superior colliculus; (4) lateral hypothalamus; (5) medial thalamus; (6) hippocampus; (7) occipital cortex and (8) cerebellar white matter. Statistical analysis by two-way ANOVA for the results in each strain, using brain regions and prion origin (*in vivo*- or *in vitro*-produced) as the variables, indicated that the differences were not significant in any of the strains studied. Conversely, the differences in the vacuolation profile among distinct strains were statistically significant ($P < 0.05$). The table on the bottom right side shows the statistical comparison among each individual strain. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

ences were also observed in the animals inoculated with PMCA-generated PrP^{Sc}. The neuropathological similarities produced by inoculation of *in vitro*- and *in vivo*-produced PrP^{Sc} were also evident by immunohistochemical staining with antibodies against PrP and GFAP (a marker for reactive astrocytes) (Figure 4).

Another difference among strains widely exploited to characterize and differentiate them is the biochemical properties of PrP^{Sc} accumulated in the brain. PrP^{Sc} associated with different strains can be distinguished biochemically by its glycosylation profile, PK cleavage, susceptibility to protease digestion, resistance to denaturation and secondary structure (Bessen and Marsh, 1992; Caughey *et al*, 1998; Safar *et al*, 1998; Hill *et al*, 2003). To assess some of these biochemical properties, we studied PrP^{Sc} from the brain of animals killed after showing signs of the disease produced by inoculation with different strains of infectious material generated *in vitro* by PMCA or obtained from the brain of sick mice. Comparison of the PrP^{Sc} western blot profile after PK digestion showed that both the glycoform distribution and the electrophoretic mobility are similar for each strain regardless of whether the infectious material was derived from brain or

generated by PMCA (Figure 5A and B). As described before, PrP^{Sc} in the mouse strains originated from scrapie have a glycoform distribution dominated by the mono-glycosylated form, whereas in PrP^{Sc} from the 301C strain the most abundant isoform is the di-glycosylated form (Figure 5A). As the PrP^C used as substrate for conversion was the same for all strains, these results indicate that PrP^{Sc} from distinct strains can preferentially recruit and convert PrP^C with particular glycosylation types. Importantly, comparing the results shown in Figures 1A and 5A, it is possible to conclude that the glycoform distribution of PrP^{Sc} is maintained *in vitro* and *in vivo* and that the PMCA-generated PrP^{Sc} from each strain can further propagate these biochemical characteristics when inoculated into wild-type mice. Finally, we studied the susceptibility of the misfolded protein from different strains to PK degradation. Partial resistance to proteolysis is one of the typical features of PrP^{Sc} and data suggest that the misfolded proteins associated with diverse strains exhibit different susceptibility to PK digestion (Bessen and Marsh, 1992). To compare the protease resistance profile, similar quantities of PrP^{Sc} from the brain of animals inoculated with various strains of either brain-derived or PMCA-generated

Figure 4 PrP^{Sc} accumulation and astroglyosis in animals inoculated with *in vitro*- or *in vivo*-generated prion strains. Pictures showing brain damage observed in sick mice inoculated with brain-derived (brain) or PMCA-generated (PMCA) prions of the three representative strains studied (139A, 79A and 301C). As a control, we showed the brain staining of animals inoculated with brain homogenate (without PrP^{Sc} seed) subjected to the same serial PMCA procedure. These animals did not show clinical signs of the disease and were killed 500 days after inoculation. (A) The profile of reactive astroglyosis in medulla as evaluated by histological staining with glial fibrillary acidic protein antibodies. (B) PrP accumulation in these animals as detected by staining the tissue with the 6H4 anti-PrP monoclonal antibody.

prions were treated for 60 min with 125, 250, 500, 750 and 1500 µg/ml of PK. The susceptibility of 301C PrP^{Sc} to PK degradation is dramatically different when compared with the ovine-derived strains. Figure 6 shows two of the ovine strains (139A and 79A), but similar results were obtained with the other strains used in this study (data not shown). Much larger concentrations of PK are required to degrade PrP^{Sc} from 301C than 139A or 79A strains as shown in the densitometric evaluation of the western blots from three independent experiments for each sample (graphs in

Figure 5 Proportion of distinct PrP^{Sc} glycoforms in animals inoculated with diverse strains of *in vivo*- and *in vitro*-generated prions. (A) Aliquots from the brain homogenate of clinically sick mice inoculated with either brain-derived (B for brain) or PMCA-generated (P for PMCA) PrP^{Sc} from different mouse strains were subjected to proteinase K (PK) digestion (50 µg/ml for 60 min at 37°C) and evaluated by western blot. All samples were digested with PK before western blot, except in the normal brain homogenate (NBH), used as a control of PrP^{Sc} migration. (B) Densitometric analysis of the western blots of three independent experiments as the one shown in (A) was done to calculate the proportion of each glycoform (di- in black bars, mono- in white bars or un-glycosylated in gray bars). The values correspond to the average ± s.e. of the three determinations. Statistical analysis by two-way ANOVA showed that none of the differences in glycoform distribution between animals inoculated with brain-derived or PMCA-generated prions was significant. Conversely, the differences in the glycoform distribution between the ovine- (RML, 139A, ME7 and 79A) and bovine-adapted (301C) strains were statistically significant ($P < 0.01$). A full-colour version of this figure is available at *The EMBO Journal* Online.

Figure 6). Importantly, PrP^{Sc} from animals inoculated with *in vitro*-generated material exhibits the same characteristics of PK resistance.

Discussion

The prion hypothesis proposes that the infectious agent responsible for TSE transmission is a misfolded protein that has the unique capability to propagate the disease by transmitting its abnormal folding properties (Prusiner, 1998). Undoubtedly, the biggest challenge of the 'protein-only' hypothesis has been to provide a credible molecular explanation to the prion strain phenomenon (Chesebro, 1998; Somerville, 2002; Aguzzi *et al*, 2007; Morales *et al*, 2007). Prions as conventional infectious agents exist in different forms to produce diseases with distinct characteristics (Bruce, 2003; Aguzzi *et al*, 2007; Morales *et al*, 2007). In traditional infectious diseases, strains are determined by polymorphisms or mutations in the nucleic acid of the agent. However, prions do not appear to contain nucleic acids. Therefore, a key question in the field has been to understand how a single protein can encode the diversity of information needed to produce and propagate many strains. The proposal that strains are dependent on differences in the folding and aggregation stage of the prion protein that can be faithfully propagated in the animal to determine diverse

Figure 6 Extent of protease resistance of PrP^{Sc} associated with different mouse prion strains. **(A)** Western blot showing the extent of PK-resistant PrP^{Sc} observed in sick mice inoculated with brain-derived (brain) or PMCA-generated (PMCA) prions from three of the strains tested: 139A (upper panel), 79A (middle panel) and 301C (lower panel). For this study, aliquots of brain homogenate were incubated for 60 min at 37°C with the indicated concentrations of PK, and PrP^{Sc} signal remaining was detected by western blot analysis as described in Materials and methods. All samples were digested with PK before western blot, except for the normal brain homogenate (NBH), used as a control of PrP^C migration. **(B)** Densitometric analyses of the western blots of three independent experiments for each strain as those shown in (A). It is important to note that the values of the graphs shown in panels of (B) represent the average of three independent experiments, explaining why sometimes the numbers do not correspond exactly with the western blots. These data enable to determine the susceptibility of PrP^{Sc} to PK from the various sources and estimate the PK₅₀ value, which corresponds to the PK concentration needed to degrade 50% of the protein. The PK₅₀ values for the experiments shown in the figure are the following: RML brain = 224 µg/ml; RML PMCA = 248 µg/ml; 79A brain = 196 µg/ml; 79A PMCA = 192 µg/ml; 301C brain = 405 µg/ml; 301C PMCA = 438 µg/ml. Statistical analysis by two-way ANOVA showed that the differences between brain and PMCA infectious material were not significant for any of the strains studied.

disease phenotypes has been taken with scepticism (Farquhar *et al*, 1998). It is hard to reconcile the last 20 years of knowledge on protein folding with the idea that a single protein can adopt dozens of stable alternative structures that can imprint their unique folding into the natively folded normal prion protein. An alternative hypothesis, termed the 'unified theory', has been proposed to suggest that although PrP^{Sc} is the infectious agent, the strain diversity is dependent on the presence of an ancillary cofactor, likely a nucleic acid (Weissmann, 1991). Although many efforts to identify such nucleic acid cofactor have failed (Kellings *et al*, 1994; Safar *et al*, 2005), the consensus hypothesis is still considered a plausible explanation for the strain phenomenon. One of the strongest arguments against the idea that strains are enciphered exclusively in the PrP^{Sc} folding is that so far the strain-specific infectious properties have not been generated or propagated *in vitro* in a cell-free system.

In a previous study, we successfully generated and propagated infectious prions *in vitro* using the PMCA technology to autocatalytically replicate PrP^{Sc} in a cell-free system (Castilla *et al*, 2005). We showed that hamster PrP^{Sc} could be maintained indefinitely replicating even in the absence of any molecule of brain-derived PrP^{Sc}. Inoculation of wild-type hamsters with *in vitro*-produced PrP^{Sc} led to a scrapie disease similar to the illness produced by brain infectious material (Castilla *et al*, 2005). Subsequently, we showed that large amounts of infectious material can be generated from sub-infectious quantities of sick hamster brain, equivalent to a single particle of PrP^{Sc} oligomers (Saá *et al*, 2006). These studies provided a strong proof in favor of the prion hypothesis, but did not address the important issue of prion strains, as only one strain of hamster PrP^{Sc} was used. Also, the *in vitro*-generated infectious agent produced disease with a delay in the incubation period, and thus the possibility that

strain characteristics were not maintained could not be ruled out. A report from Kretzschmar's group confirmed our results and using nitrocellulose immobilization experiments, showed that the delay on the disease onset was likely not due to a strain switch, but possibly to a different size proportion of PrP^{Sc} oligomers that changed their clearance rate (Weber *et al*, 2007). Our current study shows that infectious prions can be generated *in vitro* by serial replication of PrP^{Sc} misfolding in a different species as the previous experiments. Interestingly, in contrast to our previous report, the current study shows that inoculation with *in vitro*-generated prions produced the same incubation time as *in vivo*-derived infectious material. These findings further support the concept that PrP^{Sc} is the only component needed for infectivity and that *in vitro*-produced prions are very similar to the agent generated in the brain of sick animals. Nevertheless, the most relevant result of the current study is the successful propagation of the PrP^{Sc} biochemical properties of five different mouse prion strains and four human strains. Furthermore, the *in vitro*-generated mouse prion strains were infectious to wild-type mice conserving the typical properties of each strain. PrP^{Sc} from four mouse-adapted scrapie strains (RML, 139A, ME7 and 79A) and one strain with cattle origin (301C) were propagated *in vitro* by 20 successive rounds of PMCA followed by dilutions to get rid of the initial inoculum progressively. After this procedure, the sick brain used as inoculum was diluted 10²⁰-folds, and therefore no molecules of PrP^{Sc} should be present unless they were generated *in vitro* during PMCA. Our data indicate that the strain-specific differences in biochemical features of PrP^{Sc} (electrophoretic mobility and proportion of various glycoforms) associated with these strains were maintained after *in vitro* propagation, despite the fact that all the strains were replicated using the same source of PrP^C substrate. More strikingly, when administered to wild-type mice, *in vitro*-generated PrP^{Sc} produced a disease in all cases with the same clinical features and incubation time as the parental strain. A detailed study of the neuropathological and biochemical characteristics of the brain of sick animals showed that *in vitro*-generated prions produced the same profile of damage as *in vivo*-derived infectious material. These results unequivocally demonstrate that strain characteristics were replicated *in vitro* by PMCA. Our findings provide additional support for the prion hypothesis and strongly argue that strain characteristics can be faithfully propagated in the absence of living cells, suggesting that strain variation is dependent on PrP^{Sc} biochemical changes.

Materials and methods

Biological samples

Five different mouse prion strains extensively adapted and characterized were used for these experiments. All strains were propagated several times in C57Bl6 wild-type mice. Four human prion strains, associated with vCJD or sCJD (types MM1, MM2 and VV2), were obtained from patients with clinically and neuropathologically confirmed prion disease. Human PrP^{Sc} was replicated using brains of transgenic mice overexpressing human PrP with MM or VV genotype at position 129. Brains from healthy animals were extracted after mice were perfused with phosphate-buffered saline (PBS) plus 5 mM EDTA. Brain homogenates (10%, w/v) were prepared in conversion buffer (PBS containing NaCl 150 mM, 1.0% Triton X-100, 4 mM EDTA and the completeTM cocktail of protease inhibitors from Boehringer Mannheim, Mannheim, Germany).

Brain from sick individuals were harvested and homogenized in PBS plus protease inhibitors. The samples were clarified by a brief, low-speed centrifugation (2000 r.p.m. for 45 s) using an Eppendorf centrifuge (Hamburg, Germany), model 5414.

Serial replication of prions *in vitro* by PMCA

Aliquots of 10 µl of 10% brain homogenate from animals or humans infected with diverse strain were diluted into 90 µl of 10% normal brain homogenate 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, NY) and programmed to perform cycles of 30-min incubation at 37°C followed by a 20-s pulse of sonication set at potency of 7. Samples were incubated, without shaking, immersed in the water of the sonicator bath. After a round of 96 cycles, a 10 µl aliquot of the amplified material was diluted into 90 µl of normal mouse brain homogenate and a new round of 96 PMCA cycles was performed. This procedure was repeated 19 times to reach a 10⁻²⁰ final dilution of the initial sick brain homogenate. The detailed protocol for PMCA, including reagents, solutions and troubleshooting, has been published elsewhere (Castilla *et al*, 2004, 2006; Saá *et al*, 2004).

PK degradation assay

The standard procedure to digest PrP^{Sc} consists of subjecting the samples to incubation in the presence of PK (50 µg/ml) for 60 min with shaking at 37°C. The digestion was stopped by adding electrophoresis sample buffer and the protease-resistant PrP was revealed by western blotting, as indicated below. To study the profile of PK sensitivity for *in vitro*- and *in vivo*-generated PrP^{Sc}, the samples were incubated for 60 min at 37°C with different concentrations of PK ranging from 125 to 1500 µg/ml.

Western blot

Proteins were fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), electroblotted into nitrocellulose membrane and probed with 6H4 or 3F4 antibodies at a 1:5000 dilution for mouse and human samples, respectively. The immunoreactive bands were visualized by enhanced chemoluminescence assay (Amersham, Piscataway, NJ) using a UVP image analysis system. The immunoreactive bands were visualized by enhanced chemoluminescence assay (Amersham) using an UVP image analysis system. To assess the quantity of PrP^{Sc} in the western blot, densitometric analyses were done in triplicate.

Protein deglycosylation assay

PrP^{Sc} samples were first digested with PK as describe above. After addition of 10% sarkosyl, samples were centrifuged at 100 000 g for 1 h at 4°C, the supernatant was discarded and the pellet was resuspended in 100 µl of glycoprotein denaturing buffer (New England Biolabs, Beverly, MA) and incubated for 10 min at 100°C. Thereafter, 13 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) were added. Samples were incubated overnight at 37°C with shaking and the reaction was stopped by adding electrophoresis buffer and samples analysed by western blot as indicated before.

PrP^{Sc} quantification

To inject the same quantity of PrP^{Sc} from each strain preparation, both *in vitro* generated and *in vivo* produced, the samples were compared by western blotting after PK digestion. To obtain a reliable and robust quantification, we ran several different dilutions of the sample in the same gel, to avoid artefacts due to saturation of the signal or to a too weak signal.

Infectivity studies

In vivo infectivity studies were done in C57Bl6 female mice purchased from Charles River. Animals were 4–6 weeks old at the time of inoculation. Anaesthetized animals were injected stereotactically in the right hippocampus with 2 µl of the sample. The quantity of infectious material injected corresponds to the plateau portion of the incubation period (Supplementary Figure 1), therefore small differences in the amount of infectivity should not change incubation periods unless there are strain differences. To estimate the plateau of incubation period, we inoculated mice with different dilutions of infectious prions from distinct strains. The result indicates that dilutions lower than 150-fold of sick brain homo-

genate are at plateau of infectivity (Supplementary Figure 1). In our experiment, the amount of infectious material corresponds to between 15- and 30-fold dilution. The onset of clinical disease was measured by scoring the animals twice a week using the following scale: (1) normal animal; (2) roughcoat on limbs; (3) extensive roughcoat, hunchback and visible motor abnormalities; (4) urogenital lesions and (5) terminal stage of the disease in which the animal present cachexia and lies in the cage with little movement. Animals scoring level 5 were considered sick and were killed to avoid excessive pain using exposition to carbonic dioxide. Brains were extracted and analysed histologically. The right cerebral hemisphere was frozen and stored at -70°C for biochemical studies of PrP^{Sc} and the left hemisphere was used for histology analysis.

Histopathological studies

Brain tissue was fixed in 10% formaldehyde solution, embedded in paraffin and cut into sections. Serial sections (6 μm thick) from each block were stained with haematoxylin–eosin, or incubated with the 6H4 monoclonal antibody recognizing PrP or the glial fibrillary acidic protein, using our previously described protocols (Castilla *et al*, 2005). Immunoreactions were developed using the peroxidase–antiperoxidase method, following manufacturer's specifications. Antibody specificity was verified by absorption. Samples were visualized with a Zeiss microscope. The vacuolation profile was estimated by considering both number and size of vacuoles. Each analysed brain area was scored from 0 to 4 according to the

extent of vacuolation in slides stained with haematoxylin–eosin and visualized at a $\times 40$ magnification. Samples were analysed blindly by two different people and the scores represent the average of the two determinations. The brain areas studied are the following: (1) dorsal medulla; (2) cerebellar grey matter; (3) superior colliculus; (4) lateral hypothalamus; (5) medial thalamus; (6) hippocampus; (7) occipital cortex and (8) cerebellar white matter.

Statistical analysis

The quantitative differences in the vacuolation profile obtained by histopathological analysis were assessed by two-way ANOVA using brain areas and source of infectious material as the variables. Biochemical differences in PK susceptibility among strains were also analysed by two-way ANOVA using the PK concentration and source of infectious material as the variables. In both cases, the data were analysed using the GraphPad Instat, version 3.05 software.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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