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Generation of genuine prion infectivity by serial PMCA

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Abstract

Prions are the causative infectious agents of transmissible spongiform encephalopathies (TSEs). They are thought to arise from misfolding and aggregation of the prion protein (PrP). In serial transmission protein misfolding cyclic amplification (sPMCA) experiments, newly formed misfolded and proteinase K-resistant PrP (PrPres) catalysed the structural conversion of cellular prion protein (PrP^C) as efficiently as PrP^{Sc} from the brain of scrapie-infected (263K) hamsters confirming an autocatalytic misfolding cascade as postulated by the prion hypothesis. However, the fact that PrPres generated *in vitro* was associated with approximately ten times less infectivity than an equivalent quantity of brain-derived PrP^{Sc} casts doubt on the "protein-only" hypothesis of prion propagation and backs theories that suggest there are additional molecular species of infectious PrP or other agent-associated factors. By combining sPMCA with prion delivery on suitable carrier particles we were able to resolve the apparent discrepancy between the amount of PrPres and infectivity which we were then able to relate to differences in the size distribution of PrP aggregates and consecutive differences in regard to biological clearance. These findings demonstrate that we have designed an experimental set-up yielding *in vitro* generated prions that are indistinguishable from prions isolated from scrapie-infected hamster brain in terms of proteinase K resistance, autocatalytic conversion activity, and - most notably - specific biological infectivity.

Keywords: self-propagation, sPMCA, size distribution, clearance, nitrocellulose, infectivity

1. Introduction

The central event in the pathogenesis of transmissible spongiform encephalopathies (TSEs) or prion diseases is the accumulation of a misfolded aggregated isoform of the cellular prion protein (PrP^C), designated PrP^{Sc} (Prusiner, 1998) that shows relative resistance to

digestion by various proteases such as proteinase K. The structural conversion of PrP^C into a protease-resistant form (PrPres) has been modelled *in vitro* in a cell-free conversion system using purified PrP^C mixed with PrP^{Sc} extracted from brains of scrapie-infected animals (Kocisko et al., 1994; Bessen et al., 1995), giving support to the "protein-only" hypothesis of prion propagation. However, due to the low yield of newly formed PrPres, this approach did not allow direct proof of the infectious properties of the *in vitro* generated misfolded prion protein. More recently, evidence that prions solely consist of misfolded prion protein was reported by Legname et al. (2004), who were able to induce pathology and protease-resistant PrP in transgenic mice by inoculating fibrillar aggregates of recombinant mouse PrP (89-230) that were generated *in vitro* (Baskakov, 2004). However, the infectious titre of the amyloid-like PrP aggregates seemed to be low, and primary transmission was achieved only in transgenic mice with a high expression level of transgenic mouse PrP (89-230).

The protein misfolding cyclic amplification reaction (PMCA), which was originally developed by Saborio et al. (2001), provides a unique opportunity to convert large quantities of PrP^C into PrPres. Based on the assumption that prion propagation follows a mechanism of seeded aggregation (Jarrett et al., 1993; Eigen, 1996), in PMCA minute amounts of PrP^{Sc} are incubated with an excess of PrP^C in a cyclic process that consists of alternating steps of incubation and sonication. In suitable reaction conditions, PrP^{Sc} aggregates recruit new PrP molecules during the incubation step. Sonication then provides additional seeds for further aggregate growth by fragmentation of these aggregates. Amplification has also been observed without sonication (Saborio et al., 2001; Lucassen et al., 2003). However, we were able to show that efficient autocatalytic amplification requires the sonication step (Piening et al., 2005). The extent of conversion depends on the number of PMCA cycles (Saborio et al., 2001; Bieschke et al., 2004) and can be enhanced by the presence of small RNA fragments (Deleault et al., 2003).

The "protein-only" model of the prion hypothesis implies that not only the initial PrP^{Sc} used to seed the reaction but also newly formed PrPres aggregates exert a converting activity on PrP^C. In order to address this issue, we developed the serial PMCA (sPMCA) method (Bieschke et al., 2004). In fact, by combining PMCA with serial passages of the reaction mixture into fresh brain homogenate to dilute out the initial PrP^{Sc} seeds, we achieved an amplification of more than two orders of magnitude and were able to confirm an autocatalytic self-propagation of misfolded prion protein as postulated by the "prion-only" hypothesis (Bieschke et al., 2004; Piening et al., 2005). In these experiments, we observed a significant increase of infectivity in the samples obtained by serial PMCA compared to suitable dilution controls. However, the specific infectivity of these samples (i.e. infectious titre/amount of PrPres) was approximately ten times lower than the infectious titre of the brain homogenate used to start the PMCA reaction. Therefore, at the time of publication of these results (Bieschke et al., 2004) we were not certain whether the observed increase of infectivity in sPMCA-derived samples was due to *in vitro* propagation of prions or merely the result of the modification of pre-existing PrP^{Sc} particles (e.g. a higher specific infectivity of pre-existing PrP^{Sc} due to fragmentation of large aggregates). Adopting the sPMCA approach, Castilla et al. (2005) were able to subsequently amplify PrP^{Sc}/PrPres by several orders of magnitude, making it possible to clearly demonstrate the formation of new infectious units. However, the consistent phenomenon that a given quantity of sPMCA-generated PrPres is associated with a considerably lower specific infectivity than authentic brain-derived PrP^{Sc} casts doubt on the "protein-only" hypothesis of prion propagation and supports theories suggesting a hypothetical species of infectious PrP, such as PrP* (Weissmann, 2005), or further agent-associated factors essential for stabilising PrP^{Sc} structure and/or required for the conversion process.

Our current experimental investigations have led us to understand the apparent discrepancy between the amount of abnormal PrPres and infectivity, which we could relate to

differences in the size distribution of PrP aggregates generated *in vitro* and consecutive differences in regard to biological clearance (Weber et al., 2006). To analyse infectivity of sPMCA-derived PrPres with a modified size distribution and/or stability, we employed chemical cross-linking on the one hand, and adsorption to suitable carriers, such as metal beads and nitrocellulose (NC-) particles, on the other hand. Of these approaches, adsorption to NC-particles was the most effective in producing sPMCA-generated PrPres that was as infectious as authentic brain-derived PrP^{Sc}. Our findings indicate that, by combining sPMCA and adsorption to NC-particles, we have devised a cell-free technique to prepare and deliver misfolded prion protein which exhibits properties indistinguishable from PrP^{Sc} derived from brains of diseased animals, including proteinase resistance, autocatalytic converting activity and infectivity *in vivo*.

2. Material and methods

2.1. Serial transmission PMCA

Serial transmission PMCA experiments and control reactions were performed as described in detail by Piening et al. (2005). Briefly, brain homogenate (10% w/v) from terminally ill Syrian hamsters infected with scrapie strain 263K was prepared according to Saborio et al. (2001), and diluted 1:20 with similarly prepared brain homogenate extracted from healthy hamsters. Samples of 200 µl were subjected to 5× 1 s of sonication using an ultrasonic microtip probe at 40% power setting (Sonopuls 2070, Bandelin, Germany), followed by 1h of incubation at 37°C. After 10 rounds of alternated sonication and incubation (corresponding to one amplification cycle), the reaction mixture was diluted 2.5-fold into normal hamster brain homogenate. In total, 15 amplification cycles, including 10 PMCA rounds each, with 14 sequential 2.5-fold dilution steps were performed (150 rounds of PMCA). After the 15th passage, aliquots of 100 µl were collected and stored at -80°C until further use, avoiding any additional freeze-thaw cycles. For control reactions, either the

sonication step ("incubation only") or the incubation step ("sonication only") was omitted. Additionally, 200 μ l-aliquots of the initial reaction mixture were subjected to 14 sequential 2.5-fold dilution steps ("dilution only") and frozen immediately.

2.2. Western blotting and quantification of proteinase K-resistant PrP

For quantification of PrPres, samples were thawed, digested with proteinase K (PK; 100 μ g/ml; ratio of total protein: PK \approx 1 : 60) for 1 h at 37°C and subjected to Western blot analysis using the 3F4 antibody at a dilution of 1:2000 (Kascsak et al., 1987). PrP was visualised by enhanced chemiluminescence reaction (GE Healthcare, Freiburg, Germany). For quantification a Diana II luminescence imaging system and the AIDA software package (Raytest, Straubenhardt, Germany) were used. Amplification factors were determined as described by Piening et al. (2005).

2.3. In vivo infectivity studies

For *in vivo* infectivity assays sPMCA-derived PrPres and corresponding control samples were either diluted 1:10 in phosphate buffered saline (PBS) (unmodified samples) or adsorbed to nitrocellulose (NC) carrier particles prepared according to Gulle et al. (1998), which also resulted in a 1:10 dilution. Briefly, NC-sheets were dissolved in DMSO (1.25 ml/cm²) at room temperature for 10 min. Subsequently, NC-particles were precipitated by dropwise addition of 1 volume of double distilled water. Particles were centrifuged at 1620 \times g for 8 min, washed 3 times with PBS and finally re-suspended in PBS (1.25 ml/cm²). Samples (50 μ l each) were inoculated *intracerebrally* (*i.c.*) into six-week-old Syrian hamsters (9 animals per experimental group). The amount of infectivity [50% *i.c.* lethal doses (LD_{50*i.c.*})] in the inoculated samples was assayed as described by Kimberlin and Walker (1977) at the observed incubation times (*t*, in days) until terminal scrapie disease occurred, using dose-incubation curves (Kimberlin and Walker, 1986) until all of the inoculated animals succumbed to the

fatal disease. Alternatively, infectivity titres were calculated according to the method of limited dilution titration (Gregori et al., 2004) if not all of the inoculated recipients developed lethal disease. For the calculation of infectivity from incubation times, the following empirical equation was used: $\log(\text{LD}_{50i.c.}) = 0.0008 t^2 - 0.2575 t + 20.7929$ [mean error of assay: $\pm 0.4 \log(\text{LD}_{50i.c.})$]. In order to confirm scrapie infection, brains from terminally diseased animals were collected, tested for PrP^{Sc} by Western blot analysis, and used for secondary passage inoculations.

2.4. Evaluation of the size distribution of scrapie prion aggregates in brain homogenates

To obtain a robust measure of sonication-induced changes in the size distribution of PrP^{Sc} aggregates in brain homogenates, we developed a centrifugation assay using a brain homogenate from mice infected with mouse-adapted scrapie strain RML (Chandler, 1961). Firstly, in order to evaluate appropriate conditions under which the bulk of PrP^{Sc} is recovered in the pellet fraction, a 10% (wt/vol) RML brain homogenate in PBS containing 0.2% (wt/vol) SDS was digested with PK (100 µg/ml, total protein: PK \approx 1 : 60) for 1 h at 37 °C and subjected to 100,800 , 2,300, 5,900, and 13,400 \times g for 15 min, respectively, in a minifuge (Eppendorf, Hamburg, Germany). The amount of PrP^{Sc} recovered in the supernatant (S1) was then quantified using Western blot analysis.

Secondly, the effect of the sonication time on the size distribution of PrP^{Sc} aggregates in brain homogenate was analysed using the same PK-digested RML brain homogenate and subjecting it to 13,400 \times g for 15 min. 200 µl-aliquots of P1 were then either left untreated, or subjected to 5 s, 15 s, 1 min, 15 min, and 20 min sonication using an ultrasound bath (Bandelin, Berlin, Germany). Subsequently, samples were centrifuged as above, and the recovery of PK-resistant PrP in the supernatant (S2) fractions was determined by Western blot analysis.

2.5. Effect of NC-particles on the biological clearance of proteins from the brain

RK13 cells (McCarthy et al., 1963) expressing hamster PrP^C were metabolically labelled with ³⁵S-methionine and ³⁵S-cysteine (GE Healthcare, Freiburg, Germany). Subsequently, the cells were lysed prior to centrifugation for 1 min at 1,000 × g. The protein content of the supernatant was adjusted to that of a 1% mouse brain homogenate. One aliquot of the [³⁵S]-labelled cell lysate was left untreated while another aliquot was adsorbed to NC-particles prepared according to Gulle et al. (1998) for 24h under constant agitation at 4°C. NC-bound samples were diluted 10-fold in the NC-suspension prior to inoculation. Samples of 30 µl were inoculated *i.c.* into C57BL6 mice. Brains were analysed 24h post inoculation using a TRI-CARB 2900TR beta-counter (Perkin Elmer, Rodgau, Germany). As a control, dead mice were inoculated with the same aliquots.

2.6. Analysis of the infectivity of photochemically cross-linked, metal-bound, and NC-adsorbed samples

To analyse the infectivity of *in vitro* generated misfolded PrP with a modified size distribution and/or stability, sPMCA-derived PrPres and the corresponding control samples were diluted 1:10 in phosphate buffered saline (PBS) and either left unmodified (unstabilised samples), subjected to chemical crosslinking as described (Piening et al., 2006) or adsorbed to metal beads (316L, Hauner, Röttenbach, Germany). Briefly, 0.1 g metal beads and 1 ml sample were incubated for 90 min at room temperature with constant shaking. Adsorption of samples to NC-particles was conducted as above. 50 µl-aliquots of unmodified and stabilised samples were inoculated *i.c.* into the reporter animals (9 animals per group). Inoculation and clinical monitoring of hamsters were performed using blinded sample aliquots. The amount of infectivity [50% *i.c.* lethal doses (LD_{50*i.c.*})] in the inoculated samples was assayed as described by the observed incubation times (t, in days) until terminal scrapie disease occurred, using

dose-incubation curves (Kimberlin and Walker, 1986). For the calculation of infectivity from incubation times, the following empirical equation was used: $\log(\text{LD}_{50i.c.}) = 0.0008 t^2 - 0.2575 t + 20.7929$ [mean error of assay: $\pm 0.4 \log(\text{LD}_{50i.c.})$].

3. Results

3.1. *In vitro* amplification of misfolded prion protein

To test for an autocatalytic molecular conversion process, we generated PrPres by PMCA and serially passaged the reaction mixture into fresh brain homogenate to ensure a constant supply with PrP^C as a substrate for conversion (Fig. 1A). Using this serial PMCA (sPMCA) approach, we could prove an autocatalytic replication of misfolded PrPres in a cell-free conversion system (Bieschke et al., 2004). Under these conditions, the rate of PrPres generation was not matched by a corresponding increase of *in vivo* infectivity during the PMCA reaction. Therefore, we further refined the sPMCA system, and after 15 cycles of sPMCA we achieved a preparation of misfolded protein that was biochemically and structurally identical to brain-derived PrP^{Sc} but lacked any molecule of the initial scrapie-infected inoculum allowing a clear separation from the infectivity of the initial brain material used to seed the reaction (Fig. 1B). In contrast, in sPMCA reactions lacking the incubation step, the PrPres level dropped below detection limit already after the seventh 2.5-fold dilution step (Fig. 1C).

3.2. Bioassay of infectivity in hamsters

To evaluate the infectious properties of newly formed PrPres, groups of healthy Syrian hamsters were inoculated *intracerebrally* (*i.c.*) with 50 μl of sPMCA generated PrPres and corresponding controls (Weber et al., 2006). Animals inoculated with the initial reaction mixture before sPMCA ("starting material") showed a mean incubation time to terminal disease of 90 ± 3.7 days, corresponding to a mean infectious titre in the inoculum (containing

2.5 × 10⁻⁵ g of homogenised scrapie-infected hamster brain) of 1.3 × 10⁴ LD_{50i.c.} (Table 1, line "unstabilised"). Only two of nine hamsters challenged with the "dilution only" sample showed signs of scrapie at 120 and 150 days after inoculation, and none of the animals in the "no sonication"- and "no incubation"-groups became ill. This is consistent with the titre of the "starting material" group and proves that diluted control samples contained well below one infectious unit per inoculum.

The mean incubation time for the "sPMCA"-sample was 104 ± 6.0 days, corresponding to 4.6 × 10² LD_{50i.c.} per inoculum. As this sample contained the same amount of scrapie-infected brain as the diluted control samples, sPMCA resulted in an increase in infectivity by more than three orders of magnitude. Our results, therefore, corroborate the findings of Castilla et al. (2005) who have recently shown that misfolded prion protein generated by serial PMCA is associated with biological infectivity.

3.3. Size distribution of scrapie prion aggregates in brain homogenates

The fact that the apparent specific infectivity of sPMCA-derived PrP^{Sc} is more than ten times lower than for brain-derived PrP^{Sc} is consistent with recent findings (Bieschke et al., 2004; Castilla et al., 2005). We reasoned that apart from conformational differences and potential cofactors, the specific biological infectivity of PrP^{Sc} preparations ought to also depend on the size distribution of PrP^{Sc} aggregates which in turn ought to affect the number of infectious units per given amount of PrP^{Sc}. To address this issue we investigated sonication-induced changes of the size distribution of PrP^{Sc} aggregates in brain homogenates.

To obtain a robust measure of sonication-induced changes in the size distribution of PrP^{Sc} aggregates in brain homogenates, we developed a centrifugation assay using a PK-digested brain homogenate from mice infected with mouse-adapted scrapie strain RML (Fig. 2). Under appropriate conditions the bulk of PrP^{Sc} was recovered in the pellet fraction as evidenced by the decreased recovery of PrP^{Sc} in the supernatant fractions (Fig. 2A, lanes 2 -

6). The effect of the sonication time on the size distribution of scrapie prion aggregates in brain homogenates was then analysed using the same PK-digested P1 fraction and subjecting it to different sonication conditions using an ultrasound bath (Bandelin, Berlin, Germany). Figure 2B clearly demonstrates that sonication induced a dose-dependent shift towards a smaller aggregate size, as evidenced by the increased recovery of PrP^{Sc} in supernatant (S2) fractions (Fig. 2B, lanes 5 - 9). It is worth noting that when we compared the infectious titre of samples that contained the same amount of protease-resistant PrP, albeit with a different size distribution of PrP^{Sc} aggregates, we found that sonication-induced aggregate fragmentation was associated with a pronounced prolongation of incubation times (Fig. 2C). This indicates that in the murine bioassay used, measured infectivity decreased with increasing fragmentation of PrP^{Sc} aggregates.

3.4. Biological clearance of proteins from the brain

To investigate whether the sonication-induced shift towards smaller PrP^{Sc} aggregates accounts for the observed decreased biological infectivity by reducing aggregate stability and facilitating clearance from the brain, we compared ³⁵S-labelled cell lysate extracted from RK13 cells expressing hamster prion protein adsorbed to NC-particles to control samples (Fig. 3A). Whereas exactly the same amount of radioactivity remained in the brains of *i.c.* inoculated dead C57BL6 mice (Fig. 3B, white columns), in living animals, clearance of radioactivity was strongly reduced in the presence of NC-particles (Fig. 3B, black columns).

3.5. *In vivo* infectivity of sPMCA-generated modified PrPres aggregates

To analyse the infectious properties of sPMCA-derived PrPres with a modified size distribution and/or stability, we employed chemical cross-linking on the one hand, and adsorption to suitable carriers on the other hand. Applying the photo-induced PICUP cross-linking method (Fancy and Kodadek, 1999), we were able to stabilise *in vitro* generated prion

aggregates without altering their capacity to induce protein aggregation. Nevertheless, cross-linking of the samples did not eliminate the difference in the infectious titre between the starting material and the sPMCA-derived samples, whereas following adsorption to metal beads, no significant difference in infectious titres was observed (Table 1). In contrast, adsorbed to nitrocellulose particles the incubation times in the "sPMCA" group (101 ± 4.5 days, corresponding to 8.8×10^2 LD_{50i.c.}) converged to the "starting material" group (96 ± 6.0 days, corresponding to 2.8×10^3 LD_{50i.c.}). Notably, the difference in incubation times was not statistically significant ($p > 0.05$, two-tailed t-test), whereas for the corresponding unmodified samples incubation times differed significantly ($p < 0.001$). The incubation times for the starting material with and without NC were not significantly different, thus ruling out an unspecific general effect of NC-particles on incubation times.

3.6. Biochemical characterisation of diseased animals

Prion disease was confirmed by Western blot analysis of PK-digested brain homogenates extracted from terminally ill animals. No differences in regard to biochemical strain characteristics, such as electrophoretic mobility and glycoform pattern, and clinical disease phenotype were found between the different experimental groups (Fig. 4A). These findings were confirmed by secondary passage of brain homogenates from affected animals, which resulted in incubation times and a disease phenotype indistinguishable from the 263K strain used to seed the sPMCA (Fig. 4B). At 250 days after inoculation, none of the hamsters challenged with NC-adsorbed samples from the "dilution only" and "no sonication" groups developed clinical disease and only 2 animals in the "no incubation" group became ill at 209 and 226 days post infection, demonstrating that adsorption to NC-particles without PMCA is not sufficient to induce disease.

4. Discussion

According to the "prion-only" hypothesis of prion propagation the abnormal protein conformation (PrP^{Sc}) propagates in an autocatalytic manner via recruitment of the normal isoform (PrP^C) as a substrate for conversion (Prusiner, 1982). Using our PMCA approach we were able to provide proof for autocatalytic replication of misfolded PrPres in a cell-free conversion system (Bieschke et al., 2004; Piening et al., 2005). Whether the misfolded prion protein itself is the sole component of the prion infectious agent or additional cellular components are required, e.g. for stabilising PrP^{Sc} structure, and/or for conversion from PrP^C to PrP^{Sc}, requires further investigation.

The ability of *in vitro* generated PK-resistant PrPres to self-propagate in an autocatalytic manner is strong evidence in favour of the prion hypothesis. Whereas our previous experiments based on PMCA reactions with only 100 rounds of sonication/incubation led to ambiguous results in regard to amplification of infectivity (Bieschke et al., 2004), our current findings allow a clear separation of newly formed infectious units from the infectivity of the initial brain material used to seed the conversion reaction. We have generated a preparation of misfolded prion protein *in vitro*, which exhibits properties strikingly similar to those of PrP^{Sc} extracted from brains of infected animals, including proteinase resistance, autocatalytic converting activity, and *in vivo* infectivity. Our results, therefore, corroborate the findings of Castilla et al. (2005) who have recently shown that misfolded prion protein generated by serial PMCA is associated with biological infectivity. Moreover, we were able to resolve the apparent discrepancy observed in previous studies on the amount of PrPres and the titre of infectivity (Weber et al., 2006).

The fact that a given quantity of sPMCA-generated PrPres is associated with a lower infectious titre than brain-derived PrP^{Sc} could be due to a number of reasons. Apart from potential conformational differences and potential cofactors, the specific biological infectivity of PrPres preparations should also depend on the size distribution of PrPres aggregates and

their rate of clearance from the site of inoculation. This in turn should influence the level of biological infectivity per given amount of PrPres.

Using a centrifugation assay, we were clearly able to demonstrate that ultrasonic treatment breaks up aggregates of misfolded PrP into smaller units, and that sonication-induced fragmentation of prion aggregates is associated with a pronounced prolongation of incubation times in the murine bioassay used. Similarly, the relatively low apparent specific infectivity of sPMCA-derived PrPres previously reported (Bieschke et al., 2004; Castilla et al., 2005) may have resulted from fragmentation of PrP aggregates by ultrasonication during the sPMCA reaction, which in turn decreases biological infectivity by reducing aggregate stability and enhancing clearance from the brain. Therefore, we reasoned that adsorption to suitable carriers might reduce this confounding effect in the bioassay.

Of all the attempts to modify size distribution and/or stability of *in vitro* generated prion aggregates, adsorption to NC-particles turned out to be the most effective. When coupled to NC-particles, which are known to firmly retain proteins, sPMCA-generated PrPres induced clinical disease in wild-type hamsters as efficiently as the initial reaction mixture without PMCA. In contrast, NC-particles did not shorten the incubation time for brain-derived PrP^{Sc}. This important control shows that NC-particles only increased the infectious titre of samples with smaller aggregate size, thus ruling out an unspecific general effect of NC-particles on incubation times.

Thus, we reasoned that prion delivery on NC-particles reduces differences in infectivity that are primarily due to variations in the size distribution of PrPres aggregates. Shortly after *i.c.* inoculation, the major part of infectivity disappears rapidly from the brain (Safar et al., 2000). The rate of this clearance is highly variable and influenced by the particle size and the inoculum strain (Masel et al., 2001; Safar et al., 2005). The finding that PMCA leads to a shift in the size distribution of PrPres aggregates towards smaller particles is in accordance with the notion that infectious particles falling below a critical size exhibit a

higher rate of clearance. Hence, the reduced survival times in the NC-adsorbed sPMCA-derived samples are ascribed to reduced clearance from the brain rather than to structural modifications of PrPres particles induced by adsorption to nitrocellulose. This reasoning is further supported by the finding that neither sPMCA nor adsorption to NC-particles alters the biochemical strain properties of PrP^{Sc} extracted from the brains of diseased animals. Our results, therefore, do not support the previously formulated hypothesis of Castilla et al. (2005) regarding potential changes in strain properties during *in vitro* amplification of PrPres.

5. Conclusions

By combining sPMCA with prion delivery on suitable carrier particles we have devised a cell-free technique to generate misfolded prion protein *in vitro* that exhibits properties indistinguishable from authentic PrP^{Sc} extracted from the brain of diseased animals in regard to proteinase resistance, autocatalytic converting activity and infectivity *in vivo*. Using this technique, we were able to resolve the apparent discrepancy observed in previous studies regarding the amount of PrPres and infectivity. Our results provide a molecular explanation of this finding that does not require the postulation of a hypothetical maturation intermediate such as PrP* (Weissmann, 2005), as all essential properties of the infectious agent can be related to molecular properties of PrPres, provided that confounding effects related to difference in the size distribution are abrogated by prion delivery on suitable carrier particles. This in turn disproves one of the main arguments against the "protein -only" hypothesis.

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378 Part of these data have been published in Weber et al. (2006)

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Table 1

Incubation times and corresponding infectious titres for NC-adsorbed, cross-linked and metal-adsorbed samples before ("starting material") and after sPMCA ("sPMCA-derived") as well as corresponding controls ("sonication only", "incubation only", and "dilution only"). Incubation times and infectious titres are denoted by dpi and $LD50_{i.c.}$, respectively.

Figure legends

Fig. 1. Autocatalytic self-propagation of *in vitro* generated misfolded PrP.

(A) Schematic presentation of serial transmission PMCA (sPMCA). In sPMCA one amplification cycle consisting of 10 rounds of sonication and incubation was followed by a 2.5-fold dilution with uninfected brain homogenate. Diluted PrPres was again subjected to 10 rounds of sonication and incubation. This process was repeated for a total of 15 cycles (PMCA I - XV). (B) Western blot analysis of sPMCA-generated PrPres. Samples of 20 µl were taken before and after amplification, frozen for storage, digested with proteinase K and subjected to Western Blot analysis using antibody 3F4 at a 1:2,000 dilution. (C) Corresponding serial amplification reactions lacking the incubation step.

Fig. 2. Correlation between aggregate size and biological infectivity of PrP^{Sc} in C57BL/6 mice.

(A) Differential centrifugation of RML brain homogenate. A 10% (wt/vol) brain homogenate prepared from RML-infected mice was digested with proteinase K in the presence of 0.2% (wt/vol) SDS (start, lane 1), and then subjected to differential centrifugation. PrP^{Sc} contained in the supernatant (S1) after 100 × g (lane 2), 800 × g (lane 3), 2,300 × g (lane 4), 5,900 × g (lane 5), and 13,400 × g (lane 6) centrifugation was determined using 6H4 antibody at a 1:2,000 dilution. Under the conditions used, PrP^{Sc} disappeared from the supernatant fraction with increasing centrifugation speed. **(B)** Effect of the sonication time on the size distribution of PrP^{Sc} aggregates in RML brain homogenate. A 10% RML homogenate was PK-digested as above (start, lane 1). After centrifugation at 13,400 × g PrP^{Sc} was found almost exclusively in the pellet fraction (P1, lane 3). Sonication of P1 induced a dose-dependent shift towards smaller aggregate size which was evidenced by the increasing presence of PrP^{Sc} in the supernatant (S2) after 5 s (5'', lane 5), 15 s (15'', lane 6), 1 min (1', lane 7), 15 min (15', lane 8), and 20 min (20', lane 9) of ultrasonic treatment. Positions of the molecular weight markers are given on the right (in kDa). **(C)** Survival curves obtained for untreated (Ø), 5 s, and 1 min sonicated P1 samples corresponding to lanes 4, 5, and 7 in panel **(B)**.

Fig. 3. Biological clearance of proteins from mouse brains.

(A) One aliquot of a [35 S]-labelled cell lysate was left untreated (-NC) while another aliquot was adsorbed to NC-particles (+NC). Additionally, one NC-adsorbed aliquot was centrifuged for 15 min at $13,400 \times g$, and the resulting pellet was resuspended in PBS (+NC P1). Samples (-NC, +NC, +NC1 P1) of 30 μ l were inoculated *i.c.* into C57BL6 mice. (B) Retention of radioactivity in the brains of dead control mice (white columns), and in the brains of mice sacrificed 24h post-inoculation (black columns). In living animals, clearance of radioactivity was strongly reduced in the presence of NC-particles.

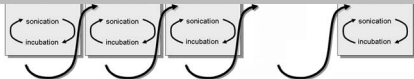
Fig. 4. Biochemical features of scrapie disease induced by inoculation of wild-type hamster with sPMCA-generated PrPres.

(A) Western blot analysis of brain homogenates extracted from diseased animals challenged with the infectious starting material ("start"), sPMCA-generated PrPres ("sPMCA"), and the initial reaction mixture after 2.5^{14} -fold dilution without PMCA ("dilut."), respectively, before ("unstabilised") and after adsorption to NC-particles ("NC-adsorbed"). (B) Secondary transmission of disease-associated prion protein in brain homogenates from symptomatic animals shown in (A) to wild-type hamsters. The brain samples examined contained large amounts of PK-resistant PrP indistinguishable to that of the 263K strain used to seed the PMCA reaction in regard to electrophoretic mobility and glycoform pattern. Positions of the molecular weight markers are given on the right (in kDa).

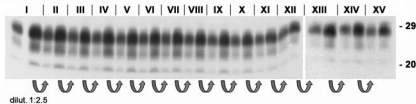
Table 1

	incubation times [days]					infectious titer [log] (corrected for PrPres content)	
	starting material	sPMCA	sonication only	incubation only	dilution only	starting material	sPMCA
unstabilsed	83	94	219	*	120	4,93	4,02
	87	99	*	*	150	4,45	3,50
	87	99	*	*	*	4,45	3,50
	91	101	*	*	*	3,99	3,31
	91	107	*	*	*	3,99	2,76
	92	107	*	*	*	3,97	2,76
	92	107	*	*	*	3,07	2,76
	94	111	*	*	*	3,66	2,43
	94	111	*	*	*	3,66	2,43
	mean ± SD	90,11 ± 3,69	104,00 ± 5,96			4,09 ± 0,43 p value (t Test) = 0,0005	3,05 ± 0,55
NC-adsorbed	87	90	209	*	*	4,45	3,81
	90	97	226	*	*	4,10	3,71
	93	98	251	*	*	3,76	3,61
	94	99	254	*	*	3,66	3,51
	94	100	*	*	*	3,66	3,41
	98	100	*	*	*	3,24	3,41
	100	103	*	*	*	3,04	3,12
	104	103	*	*	*	2,67	3,12
	104	111	*	*	*	2,67	2,43
	mean ± SD	96,00 ± 5,94	100,78 ± 4,52			3,47 ± 0,62 p-value (t-test) = 0,8406	3,35 ± 0,42
cross-linked	(49)	100	*	*	*		3,41
	86	104	*	*	*	4,56	3,03
	90	107	*	*	*	4,10	2,77
	96	107	*	*	*	3,45	2,77
	96	107	*	*	*	3,45	2,77
	96	112	*	*	*	3,45	2,35
	96	113	*	*	*	3,45	2,28
	99	113	*	*	*	3,14	2,28
	106	114	*	*	*	2,49	2,20
	mean ± SD	95,63 ± 5,90	108,56 ± 4,77			3,51 ± 0,62 p-value (t-test) = 0,0056	2,65 ± 0,41
metal-adsorbed	88	91	251	*	*	4,33	4,35
	91	101	*	*	*	3,99	3,31
	95	103	*	*	*	3,55	3,12
	96	108	*	*	*	3,45	2,68
	99	108	*	*	*	3,14	2,68
	102	109	*	*	*	2,85	2,00
	112	122	*	*	*	1,99	1,65
	112	127	*	*	*	1,99	1,36
	112	136	*	*	*	1,99	0,94
	mean ± SD	100,78 ± 9,34	111,67 ± 14,07			3,03 ± 0,89 p-value (t-test) = 0,2807	2,52 ± 1,06

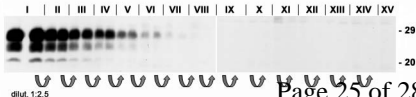
part of these data has been published in Weber et al., 2006

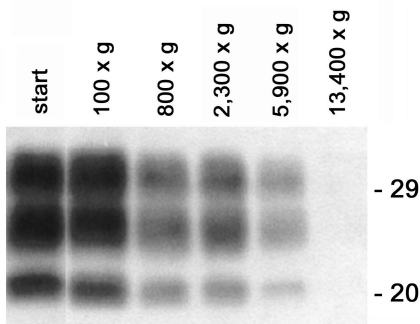


B

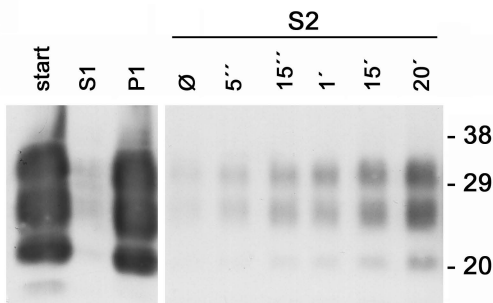


C

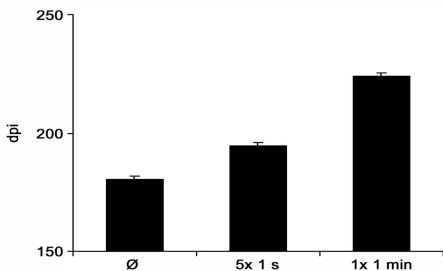


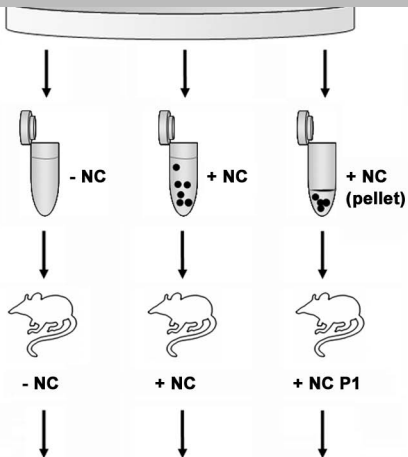


B

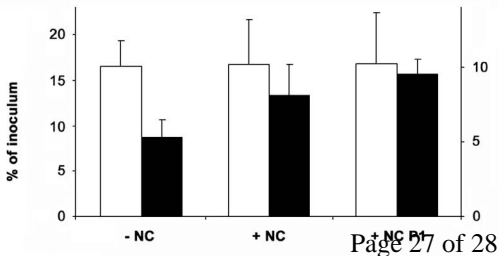


C





B



brain homogenate



sPMCA

- NC

+ NC

start

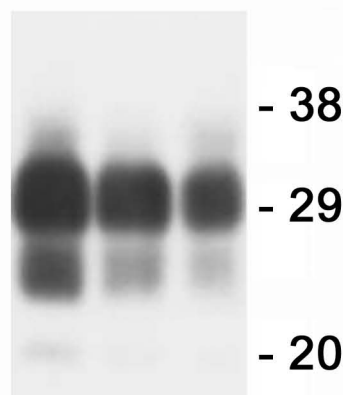
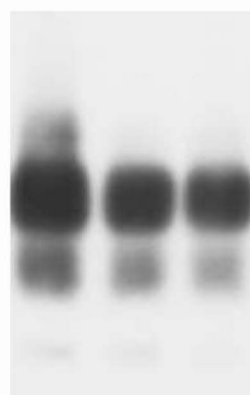
sPMCA, #1

sPMCA, #2

start

sPMCA, #1

sPMCA, #2

**primary
transmission**

start

sPMCA, #1

sPMCA, #2

sPMCA, #1

sPMCA, #2

**secondary
transmission**