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1 **Generation of genuine prion infectivity by serial PMCA**

2

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15

15 **Abstract**

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

34

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

36

37 **1. Introduction**

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

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

53 The protein misfolding cyclic amplification (PMCA), which was originally
54 developed by Saborio et al. (2001), provides a unique opportunity to convert large quantities
55 of PrP^C into PrPres. Based on the assumption that prion propagation follows a mechanism of
56 seeded aggregation (Jarrett et al., 1993; Eigen, 1996), in PMCA minute amounts of PrP^{Sc} are
57 incubated with an excess of PrP^C in a cyclic process that consists of alternating steps of
58 incubation and sonication. In suitable reaction conditions, PrP^{Sc} aggregates recruit new PrP
59 molecules during the incubation step. Sonication then provides additional seeds for further
60 aggregate growth by fragmentation of these aggregates. Amplification has also been observed
61 without sonication (Saborio et al., 2001; Lucassen et al., 2003). However, we were able to
62 show that efficient autocatalytic amplification requires the sonication step (Piening et al.,
63 2005). The extent of conversion depends on the number of PMCA cycles (Saborio et al., 2001;
64 Bieschke et al., 2004) and can be enhanced by the presence of small RNA fragments
65 (Deleault et al., 2003).

66 The "protein-only" model of the prion hypothesis implies that not only the initial PrP^{Sc}
67 used to seed the reaction but also newly formed PrPres aggregates exert a converting activity
68 on PrP^C. In order to address this issue, we developed the serial PMCA (sPMCA) method
69 (Bieschke et al., 2004). In fact, by combining PMCA with serial passages of the reaction
70 mixture into fresh brain homogenate to dilute out the initial PrP^{Sc} seeds, we achieved an
71 amplification of more than two orders of magnitude and were able to confirm an autocatalytic
72 self-propagation of misfolded prion protein as postulated by the "prion-only" hypothesis
73 (Bieschke et al., 2004; Piening et al., 2005). In these experiments, we observed a significant
74 increase of infectivity in the samples obtained by serial PMCA compared to suitable dilution
75 controls. However, the specific infectivity of these samples (i.e. infectious titre/amount of
76 PrPres) was approximately ten times lower than the infectious titre of the brain homogenate
77 used to start the PMCA reaction. Therefore, at the time of publication of these results
78 (Bieschke et al., 2004) we were not certain whether the observed increase of infectivity in
79 sPMCA-derived samples was due to *in vitro* propagation of prions or merely the result of the
80 modification of pre-existing PrP^{Sc} particles (e.g. a higher specific infectivity of pre-existing
81 PrP^{Sc} due to fragmentation of large aggregates). Adopting the sPMCA approach, Castilla et al.
82 (2005) were able to subsequently amplify PrP^{Sc}/PrPres by several orders of magnitude,
83 making it possible to clearly demonstrate the formation of new infectious units. However, the
84 consistent phenomenon that a given quantity of sPMCA-generated PrPres is associated with a
85 considerably lower specific infectivity than authentic brain-derived PrP^{Sc} casts doubt on the
86 "protein-only" hypothesis of prion propagation and supports theories suggesting a
87 hypothetical species of infectious PrP, such as PrP* (Weissmann, 2005), or further agent-
88 associated factors essential for stabilising PrP^{Sc} structure and/or required for the conversion
89 process.

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

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

103

104 **2. Material and methods**

105 *2.1. Serial transmission PMCA*

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

118 sonication step ("incubation only") or the incubation step ("sonication only") was omitted.
119 Additionally, 200 µl-aliquots of the initial reaction mixture were subjected to 14 sequential
120 2.5-fold dilution steps ("dilution only") and frozen immediately.

121

122 2.2. Western blotting and quantification of proteinase K-resistant PrP

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

130

131 2.3. *In vivo* infectivity studies

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

144 fatal disease. Alternatively, infectivity titres were calculated according to the method of
145 limited dilution titration (Gregori et al., 2004) if not all of the inoculated recipients developed
146 lethal disease. For the calculation of infectivity from incubation times, the following empirical
147 equation was used: $\log(\text{LD}_{50i.c.}) = 0.0008 t^2 - 0.2575 t + 20.7929$ [mean error of assay: ± 0.4
148 $\log(\text{LD}_{50i.c.})$]. In order to confirm scrapie infection, brains from terminally diseased animals
149 were collected, tested for PrP^{Sc} by Western blot analysis, and used for secondary passage
150 inoculations.

151

152 2.4. *Evaluation of the size distribution of scrapie prion aggregates in brain homogenates*

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

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

169

170

171 *2.5. Effect of NC-particles on the biological clearance of proteins from the brain*

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

182

183 *2.6. Analysis of the infectivity of photochemically cross-linked, metal-bound, and NC-*
184 *adsorbed samples*

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

196 dose-incubation curves (Kimberlin and Walker, 1986). For the calculation of infectivity from
197 incubation times, the following empirical equation was used: $\log(\text{LD}_{50i.c.}) = 0.0008 t^2 - 0.2575$
198 $t + 20.7929$ [mean error of assay: $\pm 0.4 \log(\text{LD}_{50i.c.})$].

199

200 **3. Results**

201 *3.1. In vitro amplification of misfolded prion protein*

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

215

216 *3.2. Bioassay of infectivity in hamsters*

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

222 2.5×10^{-5} g of homogenised scrapie-infected hamster brain) of 1.3×10^4 LD_{50*i.c.*} (Table 1, line
223 "unstabilised"). Only two of nine hamsters challenged with the "dilution only" sample showed
224 signs of scrapie at 120 and 150 days after inoculation, and none of the animals in the "no
225 sonication"- and "no incubation"-groups became ill. This is consistent with the titre of the
226 "starting material" group and proves that diluted control samples contained well below one
227 infectious unit per inoculum.

228 The mean incubation time for the "sPMCA"-sample was 104 ± 6.0 days, corresponding
229 to 4.6×10^2 LD_{50*i.c.*} per inoculum. As this sample contained the same amount of scrapie-
230 infected brain as the diluted control samples, sPMCA resulted in an increase in infectivity by
231 more than three orders of magnitude. Our results, therefore, corroborate the findings of
232 Castilla et al. (2005) who have recently shown that misfolded prion protein generated by
233 serial PMCA is associated with biological infectivity.

234

235 *3.3. Size distribution of scrapie prion aggregates in brain homogenates*

236 The fact that the apparent specific infectivity of sPMCA-derived PrP^{Sc} is more than ten
237 times lower than for brain-derived PrP^{Sc} is consistent with recent findings (Bieschke et al.,
238 2004; Castilla et al., 2005). We reasoned that apart from conformational differences and
239 potential cofactors, the specific biological infectivity of PrP^{Sc} preparations ought to also
240 depend on the size distribution of PrP^{Sc} aggregates which in turn ought to affect the number
241 of infectious units per given amount of PrP^{Sc}. To address this issue we investigated
242 sonication-induced changes of the size distribution of PrP^{Sc} aggregates in brain homogenates.

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

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

259

260 3.4. Biological clearance of proteins from the brain

261 To investigate whether the sonication-induced shift towards smaller PrP^{Sc} aggregates
262 accounts for the observed decreased biological infectivity by reducing aggregate stability and
263 facilitating clearance from the brain, we compared ³⁵S-labelled cell lysate extracted from
264 RK13 cells expressing hamster prion protein adsorbed to NC-particles to control samples (Fig.
265 3A). Whereas exactly the same amount of radioactivity remained in the brains of *i.c.*
266 inoculated dead C57BL6 mice (Fig. 3B, white columns), in living animals, clearance of
267 radioactivity was strongly reduced in the presence of NC-particles (Fig. 3B, black columns).

268

269 3.5. *In vivo* infectivity of sPMCA-generated modified PrPres aggregates

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

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

285

286 *3.6. Biochemical characterisation of diseased animals*

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

298

299 **4. Discussion**

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

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

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

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

327 Using a centrifugation assay, we were clearly able to demonstrate that ultrasonic
328 treatment breaks up aggregates of misfolded PrP into smaller units, and that sonication-
329 induced fragmentation of prion aggregates is associated with a pronounced prolongation of
330 incubation times in the murine bioassay used. Similarly, the relatively low apparent specific
331 infectivity of sPMCA-derived PrPres previously reported (Bieschke et al., 2004; Castilla et al.,
332 2005) may have resulted from fragmentation of PrP aggregates by ultrasonication during the
333 sPMCA reaction, which in turn decreases biological infectivity by reducing aggregate
334 stability and enhancing clearance from the brain. Therefore, we reasoned that adsorption to
335 suitable carriers might reduce this confounding effect in the bioassay.

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

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

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

358

359 **5. Conclusions**

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

372

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377

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

379

380 **References**

381 Baskakov, I.V., 2004. Autocatalytic conversion of recombinant prion proteins displays a
382 species barrier. *J. Biol. Chem.* 279, 7671-7677.

383 Bessen, R.A., Kocisko, D.A., Raymond, G.J., Nandan, S., Lansbury, P.T., Caughey, B., 1995.
384 Non-genetic propagation of strain-specific properties of scrapie prion protein. *Nature*
385 375, 698-700.

386 Bieschke, J., Weber, P., Sarafoff, N., Beekes, M., Giese, A., Kretzschmar, H.A., 2004.
387 Autocatalytic self-propagation of misfolded prion protein. *Proc. Natl. Acad. Sci. USA*
388 101, 12207-12211.

389 Castilla, J., Saá, P., Hetz, C., Soto, C., 2005. *In vitro* generation of infectious scrapie prions.
390 *Cell* 121, 195-206.

391 Chandler, R.L., 1961. Encephalopathy in mice produced by inoculation with scrapie brain
392 material. *Lancet* 1, 1378-1379.

393 Deleault, N.R., Lucassen, R.W., Supattapone, S., 2003. RNA molecules stimulate prion
394 protein conversion. *Nature* 425, 717-720.

395 Eigen, M., 1996. Prionics or the kinetic basis of prion disease. *Biophys. Chem.* 63, A1-A18.

396 Ertmer, A., Gilch, S., Yun, S.W., Flechsig, E., Klebl, B., Stein-Gerlach, M., Klein, M.A.,
397 Schätzl, H. M., 2004. The tyrosine kinase inhibitor STI571 induces cellular clearance of
398 PrP^{Sc} in prion-infected cells. *J. Biol. Chem.* 279, 41918-41927.

399 Fancy, D.A., Kodadek, T., 1999. Chemistry for the analysis of protein-protein interactions:
400 rapid and efficient cross-linking triggered by long wavelength light. *Proc. Natl. Acad.*
401 *Sci. USA* 96, 6020-6024.

- 402 Gregori, L., McCombie, N., Palmer, D., Birch, P., Sowemimo-Coker, S.O., Giulivi, A.,
403 Rohwer, R.G., 2004. Effectiveness of leucoreduction for removal of infectivity of
404 transmissible spongiform encephalopathies from blood. *Lancet* 364, 529-531.
- 405 Gulle, H., Eibl, M.M., Wolf, H.M., 1998. Nitrocellulose particles adsorbed to
406 immunoglobulins are a new and effective approach to induce cell activation dependent
407 on receptor aggregation. *J. Immunol. Meth.* 214, 199-208.
- 408 Jarrett, J.T., Lansbury, P.T. Jr., 1993. Seeding 'one-dimensional crystallization' of amyloid: a
409 pathogenic mechanism in Alzheimer's disease and Scrapie. *Cell* 73, 1055-1058.
- 410 Kasczak, R.J., Rubenstein, R., Merz, P.A., Tonna-DeMasi, M., Fersko, R., Carp, R.I.,
411 Wisniewski, H.M., Diringer, H., 1987. Mouse polyclonal and monoclonal antibody to
412 scrapie-associated fibril proteins. *J. Virol.* 61, 3688-3693.
- 413 Kimberlin, R.H., Walker, C.A., 1977. Characteristics of a short incubation model of scrapie in
414 the golden hamster. *J. Gen. Virol.* 34, 295-304
- 415 Kimberlin, R.H., Walker, C.A., 1986. Pathogenesis of scrapie (strain 263K) in hamsters
416 infected intracerebrally, intraperitoneally or intraocularly. *J. Gen. Virol.* 67, 255-263.
- 417 Kocisko, D.A., Priola, S.A., Raymond, G.J., Chesebro, B., Lansbury, P.T. Jr., Caughey, B.,
418 1994. Cell-free formation of protease-resistant prion protein. *Nature* 370, 471-474.
- 419 Legname, G., Baskakov, I.V., Nguyen, H.O., Riesner, D., Cohen, F.E., DeArmond, S.J.,
420 Prusiner, S.B., 2004. Synthetic mammalian prions. *Science* 305, 673-676.
- 421 Lucassen, R., Nishina, K., Supattapone, S., 2003. *In vitro* amplification of protease-resistant
422 prion protein requires free sulfhydryl groups. *Biochem.* 42, 4127-4135.
- 423 Masel, J., Jansen, V.A.A., 2001. The measured level of prion infectivity varies in a
424 predictable way according to the aggregation state of the infectious agent. *Biochim.*
425 *Biophys. Acta* 1535, 164-173.

- 426 Piening, N., Weber, P., Giese, A., Kretzschmar, H., 2005. Breakage of PrP aggregates is
427 essential for efficient autocatalytic propagation of misfolded prion protein. *Biochem.*
428 *Biophys. Res. Commun.* 326, 339-343.
- 429 Piening, N., Weber, P., Högen, T., Beekes, M., Kretzschmar, H., Giese, A., 2006. Photo-
430 induced crosslinking of prion protein oligomers and prions. *Amyloid* 13, 67-77.
- 431 Prusiner, S.B., 1982. Novel proteinaceous infectious particles cause scrapie. *Science* 216,
432 136-144.
- 433 Prusiner, S.B., 1998. Prions. *Proc. Natl. Acad. Sci. USA* 95, 13363-13383.
- 434 McCarthy, K., Taylor-Robinson, C.H., Pillinger, S.E., 1963. Isolation of rubella virus from
435 cases in Britain. *Lancet* 15, 593-598.
- 436 Saborio, G.P., Permanne, B., Soto, C., 2001. Sensitive detection of pathological prion protein
437 by cyclic amplification of protein misfolding. *Nature* 411, 810-813.
- 438 Safar, J.G., Cohen, F.E., Prusiner, S.B., 2000. Quantitative traits of prion strains are
439 enciphered in the conformation of the prion protein. *Arch. Virol. Suppl.* 16, 227-235.
- 440 Safar, J.G., Kellings, K., Serban, A., Groth, D., Cleaver, J.E., Prusiner, S.B., Riesner, D.,
441 2005. Search for a prion-specific nucleic acid. *J. Virol.* 79, 10796-10806.
- 442 Weber, P., Giese, A., Piening, N., Mitteregger, G., Thomzig, A., Beekes, M., Kretzschmar,
443 H.A., 2006. *Proc. Natl. Acad. Sci. USA*, Oct 9; Epub ahead of print.
- 444 Weissmann, C., 2005. Birth of a prion: spontaneous generation revisited. *Cell* 122, 165-168.
445

445 **Table 1**

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

450

451

452

452 **Figure legends**453 **Fig. 1. Autocatalytic self-propagation of *in vitro* generated misfolded PrP.**

454 (A) Schematic presentation of serial transmission PMCA (sPMCA). In sPMCA one
455 amplification cycle consisting of 10 rounds of sonication and incubation was followed by a
456 2.5-fold dilution with uninfected brain homogenate. Diluted PrPres was again subjected to 10
457 rounds of sonication and incubation. This process was repeated for a total of 15 cycles
458 (PMCA I - XV). (B) Western blot analysis of sPMCA-generated PrPres. Samples of 20 μ l
459 were taken before and after amplification, frozen for storage, digested with proteinase K and
460 subjected to Western Blot analysis using antibody 3F4 at a 1:2,000 dilution. (C)
461 Corresponding serial amplification reactions lacking the incubation step.

462

462 **Fig. 2. Correlation between aggregate size and biological infectivity of PrP^{Sc} in C57BL6**
463 **mice.**

464 **(A)** Differential centrifugation of RML brain homogenate. A 10% (wt/vol) brain homogenate
465 prepared from RML-infected mice was digested with proteinase K in the presence of 0.2%
466 (wt/vol) SDS (start, lane 1), and then subjected to differential centrifugation. PrP^{Sc} contained
467 in the supernatant (S1) after 100 × g (lane 2), 800 × g (lane 3), 2,300 × g (lane 4), 5,900 × g
468 (lane 5), and 13,400 × g (lane 6) centrifugation was determined using 6H4 antibody at a
469 1:2,000 dilution. Under the conditions used, PrP^{Sc} disappeared from the supernatant fraction
470 with increasing centrifugation speed. **(B)** Effect of the sonication time on the size distribution
471 of PrP^{Sc} aggregates in RML brain homogenate. A 10% RML homogenate was PK-digested as
472 above (start, lane 1). After centrifugation at 13,400 × g PrP^{Sc} was found almost exclusively in
473 the pellet fraction (P1, lane 3). Sonication of P1 induced a dose-dependent shift towards
474 smaller aggregate size which was evidenced by the increasing presence of PrP^{Sc} in the
475 supernatant (S2) after 5 s (5'', lane 5), 15 s (15'', lane 6), 1 min (1', lane 7), 15 min (15', lane
476 8), and 20 min (20', lane 9) of ultrasonic treatment. Positions of the molecular weight markers
477 are given on the right (in kDa). **(C)** Survival curves obtained for untreated (∅), 5 s, and 1 min
478 sonicated P1 samples corresponding to lanes 4, 5, and 7 in panel **(B)**.

479

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

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

487

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

489 **(A)** Western blot analysis of brain homogenates extracted from diseased animals challenged
490 with the infectious starting material ("start"), sPMCA-generated PrPres ("sPMCA"), and the
491 initial reaction mixture after 2.5¹⁴-fold dilution without PMCA ("dilut."), respectively, before
492 ("unstabilised") and after adsorption to NC-particles ("NC-adsorbed"). **(B)** Secondary
493 transmission of disease-associated prion protein in brain homogenates from symptomatic
494 animals shown in **(A)** to wild-type hamsters. The brain samples examined contained large
495 amounts of PK-resistant PrP indistinguishable to that of the 263K strain used to seed the
496 PMCA reaction in regard to electrophoretic mobility and glycoform pattern. Positions of the
497 molecular weight markers are given on the right (in kDa).

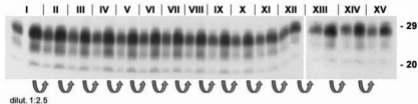
Table 1

	incubation times [days]					infectious titer [log] (corrected for PrPres content)	
	starting material	sPMCA	sonication only	incubation only	dilution only	starting material	sPMCA
unstabilised	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	*	*	*	3,87	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	
NC-adsorbed	87	96	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	
cross-linked	(48 I)	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
	98	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	
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	2,52 ± 1,06
						p-value (t-test) = 0,2807	

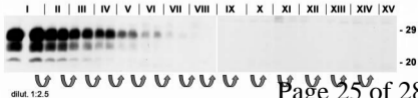
part of these data has been published in Weber et al., 2006

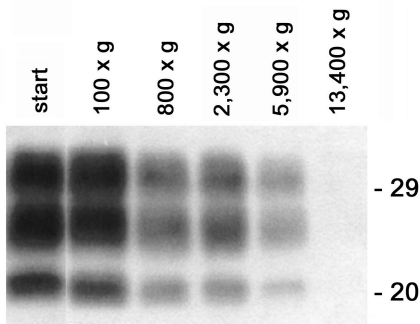
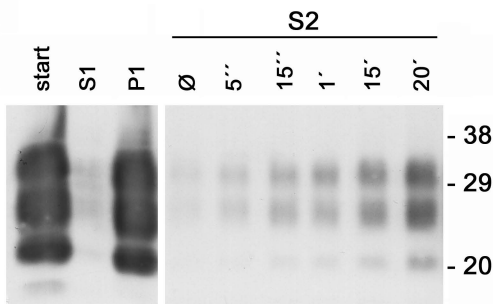
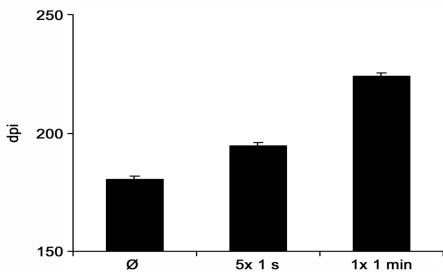


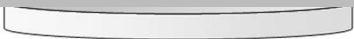
B



C



**B****C**



- NC



+ NC



+ NC
(pellet)



- NC



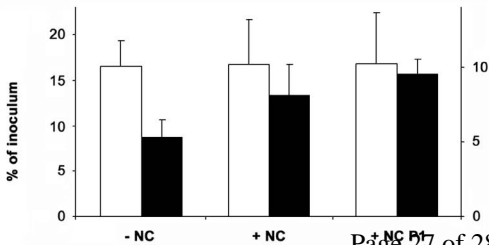
+ NC



+ NC P1



B



brain homogenate

sPMCA

- NC

+ NC

start

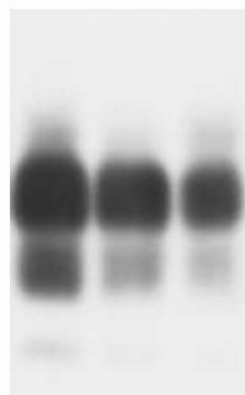
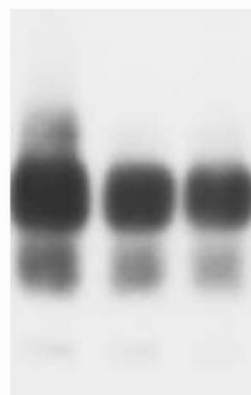
sPMCA, #1

sPMCA, #2

start

sPMCA, #1

sPMCA, #2

primary
transmission

start

sPMCA, #1

sPMCA, #2

sPMCA, #1

sPMCA, #2

secondary
transmission