Protease-resistant prion protein produced in vitro lacks detectable infectivity

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The ‘protein-only’ hypothesis of prion propagation argues that infectious prions consist of PrPSc, a conformational isomer of host-derived prion protein (PrP), which can be distinguished from PrPC by its partial resistance to proteases. While protease-resistant PrP has been produced by mixing PrPSc and recombinant-derived PrP in vitro, bioassay of any new infectivity has been precluded by the need to use a large molar excess of same species PrPSc. Transgenic mice expressing a chimaeric hamster–mouse PrPC (MH2M PrP) are, unlike conventional mice, highly susceptible to Sc237 hamster scrapie. In addition, they produce MH2M PrPSc and infectivity which is pathogenic for conventional mice. We have therefore attempted to produce MH2M PrPSc in vitro as any infectivity produced could be distinguished from the hamster PrPSc used to promote the conversion by bioassay in conventional mice. Although protease-resistant MH2M PrP was produced, no infectivity was detected on bioassay. These results argue that acquisition of protease resistance by PrPSc is not sufficient for the propagation of infectivity.

Prion diseases, such as Creutzfeldt–Jakob disease in humans and scrapie and bovine spongiform encephalopathy in animals, are associated with the accumulation of a disease-specific isoform of cellular prion protein (PrPC), designated PrPSc, which appears to be the principal or sole component of the transmissible agent or prion (Prusiner, 1991). PrPSc is derived post-translationally from its cellular precursor, PrPC, and it is hypothesized that PrPSc binds cellular PrPC and templates its conversion to further PrPSc. PrPSc is distinguished from PrPC by its physico-chemical properties, in particular by its partial resistance to proteolysis. The ‘protein-only’ hypothesis for prion propagation implies that it ought to be possible to generate prions in vitro from highly purified, recombinant-derived PrP. An important milestone was the demonstration that protease-resistant PrP could be generated in vitro by mixing hamster PrPSc with a large excess of hamster PrPSc under suitable conditions (Kocisko et al., 1994). In addition, such ‘conversion’ shows both the species specificity and strain selection characteristic of prion propagation (Bessen et al., 1995; Kocisko et al., 1995). However, while protease resistance is a key marker of prions, a number of experiments appear to uncouple infectivity and protease resistance (Xi et al., 1992; Collinge et al., 1995; Lasmezas et al., 1997; Hsiao et al., 1990, 1994). It is therefore crucial to determine by bioassay whether such protease-resistant material produced in vitro is actually infectious. The terminology PrPSc has been suggested to designate infectious PrP, which may or may not be protease resistant (Weissmann, 1991). However, the requirement to use a large molar excess of PrPSc from the same species to drive such in vitro conversion has so far precluded bioassay of any newly produced infectious agent.

Conventional mice are typically highly resistant to infection with hamster prions. This ‘species barrier’ to infection of mice with hamster prions is ablated in transgenic mice expressing a chimaeric PrP in which the central region of mouse PrP (residues 94–188) is replaced by the corresponding hamster PrP sequence (Scott et al., 1993). This chimaeric PrP is designated MH2M PrP. Such transgenic mice are susceptible to hamster prions and produce chimaeric prions which are pathogenic for wild-type mice as well as for hamsters. We therefore sought to utilize this chimaeric PrP as a means to bridge the hamster–mouse species barrier. If hamster PrPSc were able to convert MH2M PrPSc to MH2M PrPSc in vitro, then any newly produced chimaeric prions could now be effectively detected in bioassay, as they would be expected to be pathogenic for wild-type mice, unlike the large molar excess of hamster PrPSc used to promote the conversion. We therefore produced recombinant MH2M PrP in cell culture and subjected it to the same methods established by Kocisko et al. (1994) for in vitro production of protease-resistant hamster PrP.
Firstly, we confirmed the finding of Kocisko et al. that Syrian hamster PrP \(^{Sc}\) (SHAPrP\(^{Sc}\)) can be reversibly denatured in 3 M guanidinium hydrochloride with the recovery of its protease resistance on dilution (data not shown). Syrian hamsters were inoculated with the Sc237 strain of hamster scrapie and were culled in the terminal stages of disease. Sc237 has similar properties to the 263K strain of hamster scrapie (Scott et al., 1989). SHAPrP\(^{Sc}\) was purified as previously described (Kocisko et al., 1994). The final pellet was sonicated into 1% sarcosyl in PBS and the protein concentration adjusted to 1 mg/ml. Purified SHAPrP\(^{Sc}\) was reversibly denatured according to the method of Kocisko et al. in 3 M guanidinium hydrochloride. Briefly, 2 µl of SHAPrP\(^{Sc}\) was mixed with 2 µl 6 M guanidinium hydrochloride and incubated at 37 °C for between 2 and 16 h. To renature the PrP\(^{Sc}\) this mixture was diluted to 1:5 M guanidinium hydrochloride by the addition of 4 µl TN buffer (50 mM Tris–HCl pH 7.4, 100 mM NaCl) and incubated at 37 °C for up to 2 days. The concentration of guanidinium hydrochloride was then lowered to 0.075 M by the addition of TN buffer. To assess the renaturation of the PrP\(^{Sc}\) the mixture was treated with 50 µg/ml proteinase K (final concentration) for 1 h at 37 °C and the recovery of protease-resistant material (absent at 3 M guanidinium hydrochloride) was detected by Western blotting using anti-PrP antibody 3F4 (Kocisko et al., 1987).

We expressed MH2M PrP in murine erythroleukaemia cells under the control of the \(\beta\)-globin gene promoter and locus control region (Antoniou, 1991) (Fig. 1). The MH2MPPrP construct was produced as described previously (Scott et al., 1992) and the correct sequence confirmed using an Applied Biosystems 373A automated DNA sequencer. The MH2MPPrP fragment was then subcloned into the expression vector pEV.puro (Needham et al., 1995). Murine erythroleukaemia (MEL) C88 cells in the exponential phase were transfected with pEV.puro.MH2M by electroporation (Antoniou, 1991). Expressing clones were selected with puromycin and expression of MH2MPPrP was determined by Western blotting of lysates from cells which had been induced to undergo erythroid differentiation for 4 days in the presence of 2% DMSO. Blots were probed with monoclonal antibody 3F4, which detects MH2M PrP (which has the 3F4 epitope present in hamster PrP) but not mouse PrP (Kascak et al., 1987). MEL cells were labelled at day 4 after induction as follows: Each 30 ml culture was spun at 1200 g for 5 min and the cells were washed in PBS before resuspension in methionine-free \(\alpha\)-MEM media (Gibco) supplemented with glutamine (2 mM, Sigma) and penicillin–streptomycin (Sigma). Following incubation at 37 °C for 1 h, the cells were resuspended in methionine-free \(\alpha\)-MEM media (supplemented as above) and 250–500 µCi \(^{35}\)S)methionine (Promix; Amersham) added per flask equivalent of cells and incubated at 37 °C for 5 h. Cells were washed in PBS and lysed in lysis buffer (150 mM NaCl, 50 mM Tris–HCl pH 7.5, 1% Nonidet P-40, 0.5% sodium deoxycholate). Cellular debris was removed by centrifugation at 1000 g for 10 min. Monoclonal antibody 3F4 was added at a dilution of 1:500 (antibody: lysate) and the mixture was incubated at 4 °C overnight and then transferred to a fresh tube containing 50 µl of a 10% slurry of protein A–Sepharose (Pharmacia) and incubated with shaking for 1 h at room temperature. The beads were pelleted by a 1 min spin in a microfuge at 1000 g and washed in lysis buffer. The beads were resuspended in 10–20 µl SDS–PAGE loading buffer or 3 M guanidinium hydrochloride (for conversion reactions) and incubated for 30 min at 37 °C before removal of the supernatant to a fresh tube.

For the conversion reaction, purified SHAPrP\(^{Sc}\) was reversibly denatured as described above. Immunoprecipitated PrP\(^{Sc}\) was eluted from the protein A beads in 3 M guanidinium hydrochloride at 37 °C for 30 min; 2 µl of each protein were mixed and the concentration of guanidinium hydrochloride was reduced to 1:5 M by the addition of TN buffer. The mixture was incubated at 37 °C for 2 days before further dilution of the guanidinium to 0.075 M with TN buffer. The mixture was treated with proteinase K (50 µg/ml final concentration) for 1 h at 37 °C. This reaction was terminated...
by the addition of Pefabloc (Boehringer Mannheim) to a final concentration of 1 mM. 5 μg thyroglobulin was added to the mixture as a carrier protein and 4 vols of ice cold methanol were added to precipitate the proteins. The pellet was resuspended in SDS loading buffer and analysed by SDS–PAGE and the gels developed for fluorographic detection of $^{35}$S-labelled proteins (Amplify; Amersham). The gel was dried and exposed to Biomax MR (Kodak) film. This confirmed the presence of radiolabelled, protease-resistant PrP, implying recruitment of MH2M PrP into a protease resistant form (Fig. 2).

Bioassay of this material, and appropriate control reactions, were performed in Swiss CD-1 mice (Table 1). Ten reactions as above of in vitro conversion products were pooled and resuspended in 500 μl sterile PBS; 30 μl aliquots of conversion products or control reactions were inoculated intracerebrally into Swiss CD-1 mice under halothane anaesthesia. The animals were examined twice weekly for signs of neurological dysfunction. No scrapie-like disease was noted in any inoculation group at over 550 days. Groups of the same colony of CD-1 mice inoculated with mouse-passaged RML prions all developed scrapie at around 140 days. All animals which were culled as a result of intercurrent illness or which were found dead were examined neuropathologically and there was no evidence of prion disease in these mice.

Prions generated in mice transgenic for MH2M PrP in response to challenge with Sc237 hamster prions have an incubation period of around 180 days in Swiss CD-1 mice (Scott et al., 1993). Bioassay in susceptible animals is known to be able to detect infectivity, which may be present at over 10⁹ infectious units/g in affected brain (Prusiner, 1987), at serial dilutions well beyond the limit of detection of PrPSc by conventional blotting techniques (Caughey et al., 1997). Indeed, it has been estimated that a single infectious unit equates to 10⁸–10⁹ PrP molecules, or approximately 0.5–5 fg, which would be well below the detection limit of these SDS fluorographed gels (Prusiner et al., 1982; Weissmann, 1991). It therefore seems unlikely that the protease resistant MH2M PrP produced in vitro represents homogeneous authentic PrPSc.

A substantial array of experimental data supports the protein-only hypothesis for prion propagation, both with respect to the infectivity (Prusiner, 1991; Bueler et al., 1993) and strain specificity (Collinge et al., 1996; Telling et al., 1996) of prion isolates. The demonstration of both species specificity (Kocisko et al., 1995) and strain selectivity (Bessen et al., 1995) of production of protease-resistant PrP by in vitro mixing experiments argues against the labelled recombinant-derived PrPSc simply being protected from proteolysis by association with the large molar excess of PrPSc (Pan et al., 1993). Our results argue that the acquisition by PrPSc of protease resistance in vitro is not sufficient for the production of infectivity.

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### References


### Table 1. Bioassay of reaction mixtures and controls in Swiss CD-1 mice

<table>
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<tr>
<th>Inocula</th>
<th>Affected/ inoculated</th>
<th>Incubation period (days)</th>
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<tr>
<td>MH2M PrPSc/Sha PrPSc (PK +)</td>
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<td>&gt; 550</td>
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<tr>
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<td>&gt; 550</td>
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<td>Untreated Sha PrPSc</td>
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