Removal of the glycosylphosphatidylinositol anchor from PrP^{Sc} by cathepsin D does not reduce prion infectivity

Patrick A. LEWIS*, Francesca PROPERZI*, Kanella PRODRAMIDOU*, Anthony R. CLARKE†, John COLLINGE* and Graham S. JACKSON*¹

*MRC Prion Unit, Department of Neurodegenerative Disease, Institute of Neurology, University College London, Queen Square, London WC1N 3BG, U.K., and †Department of Biochemistry, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, U.K.

According to the protein-only hypothesis of prion propagation, prions are composed principally of PrP^{Sc}, an abnormal conformational isoform of the prion protein, which, like its normal cellular precursor (PrP^C), has a GPI (glycosylphosphatidylinositol) anchor at the C-terminus. To date, elucidating the role of this anchor on the infectivity of prion preparations has not been possible because of the resistance of PrP^{Sc} to the activity of PI-PLC (phosphoinositide-specific phospholipase C), an enzyme which removes the GPI moiety from PrP^C. Removal of the GPI anchor from PrP^{Sc} requires denaturation before treatment with PI-PLC, a process that also abolishes infectivity. To circumvent this problem, we have removed the GPI anchor from PrP^{Sc} in RML (Rocky Mountain Laboratory)-prion-infected murine brain homogenate using the

aspartic endoprotease cathepsin D. This enzyme eliminates a short sequence at the C-terminal end of PrP to which the GPI anchor is attached. We found that this modification has no effect (i) on an *in vitro* amplification model of PrP^{Sc}, (ii) on the prion titre as determined by a highly sensitive N2a-cell based bioassay, or (iii) in a mouse bioassay. These results show that the GPI anchor has little or no role in either the propagation of PrP^{Sc} or on prion infectivity.

Key words: cathepsin D, Creutzfeldt–Jakob disease (CJD), glycosylphosphatidylinositol anchor (GPI anchor), prion, prion infectivity.

INTRODUCTION

The prion diseases of humans and animals are a group of closely related fatal neurodegenerative disorders that include scrapie in sheep, BSE (bovine spongiform encephalopathy) in cattle and CJD (Creutzfeldt–Jakob disease), kuru, GSS (Gerstmann–Sträussler–Scheinker syndrome) and FFI (fatal familial insomnia) in humans [1]. Prion diseases in humans have three distinct aetiologies: being inherited, acquired by exposure to infectious material or arising sporadically. They are characterized by accumulation of a misfolded endogenous protein, PrP (prion protein) [2], and the protein-only hypothesis [3,4] states that the central event in the pathogenesis of these diseases is the conversion of the normal host prion protein, PrP^C, into an aberrantly folded infectious form, denoted PrP^{Sc} [5]. Although much research has been directed at unravelling the mechanism whereby this conversion occurs, very little is known about the molecular basis of PrP^{Sc} formation.

Human PrP is a 253-amino-acid protein produced from a single exon located on chromosome 20 [6]. Following translation, PrP is modified by the formation of an internal disulphide bond between residues 179 and 214 [7], glycosylation at Asp¹⁸¹ and Asp¹⁹⁷, which has now been characterized [8], and, following the removal of 22 C-terminal amino acids, the addition of a GPI (glycosylphosphatidylinositol) anchor to the C-terminal serine residue [9]. Cellular PrP is attached to the plasma membrane via this GPI anchor and is localized, along with other GPI-anchored proteins, to cholesterol-rich lipid rafts within the membrane [10]. The GPI anchor attached to PrP^C can be cleaved off by treatment of the protein with PI-PLC (phosphoinositide-specific phospholipase C), resulting in the release of the protein from the cell surface

of intact cells [11]. This also results in an apparent increase in the molecular mass of PrP, as analysed by SDS/PAGE, owing to the extremely hydrophobic nature of the lost anchor [12–15]. It has been shown in cell-culture models that location of PrP^C at the cell membrane is crucial both for infection of cells with PrP^{Sc} and for the continued propagation of the PrP^{Sc} isoform. Treatment of cell cultures with PI-PLC before the addition of infectious homogenates has a protective effect, and treatment following establishment of infection cures the cells [16]. Previous studies have demonstrated that the development of prion disease is critically dependent upon PrP expression [17,18]. A more recent study has refined this dependence to the presence of GPI-anchored cell-surface PrP: Chesebro et al. [19] generated a transgenic mouse line that expresses anchorless secreted PrP. When infected with the RML (Rocky Mountain Laboratory) strain of mouse prions, the mice produce widespread deposits of PrP^{Sc} and yet do not develop any clinical syndrome. The reasons for this have yet to be determined, but the results suggest that pathology results from effects of PrP^{Sc} formation at the surface of the cell membrane.

Analysis of the impact of GPI loss on the infectivity of PrP^{Sc} has, to date, not been possible because of the resistance of the PrP^{Sc} isoform to the enzymatic activity of PI-PLC, thought to be due to steric exclusion of the enzyme by the altered conformation of PrP [20]. It has also been shown that mutations found in inherited prion disease, when introduced into PrP expressed in cultured cell lines, increase the resistance to cleavage with PI-PLC [14]. Removal of the GPI anchor from PrP^{Sc} requires denaturation before treatment with PI-PLC, a process that also abolishes infectivity; it has thus been impossible to ascertain whether the GPI anchor is an essential component of prion infectivity. Answering

Abbreviations used: ELISPOT, enzyme-linked immunospot plate; GPI, glycosylphosphatidylinositol; OFCS, Opti-MEM® with 10% foetal calf serum; PBST, PBS containing 0.05% (v/v) Tween 20; PI-PLC, phosphoinositide-specific phospholipase C; PK, proteinase K; PrP, prion protein; PrP^C, cellular PrP isoform; PrP^{Sc}, pathogenic (scrapie) PrP isoform; RML, Rocky Mountain Laboratory; RMM, relative molecular mass.

¹ To whom correspondence should be addressed (email g.s.jackson@prion.ucl.ac.uk).

this question has several consequences regarding our understanding of prion diseases. From a mechanistic point of view, the impact of GPI removal on the ability of PrP^{Sc} to infect cells may shed light both on the site of the conversion event and on the route that the PrP^{Sc} isoform takes into the cell. Also, should the absence of a GPI anchor decrease the infectivity of PrP^{Sc}, this has implications for attempts to generate infectious prions *in vitro* from recombinant material, since the majority of recombinant prion protein is generated from bacterial sources and lacks a GPI anchor. The publication of results describing the production of putative prion infectivity from recombinant PrP [21] has led to considerable debate within the field. A demonstration that material lacking an anchor can be infectious lends credence to the possibility of recombinant prion infectivity.

In the present paper, we describe the removal of the GPI anchor from PrP^{Sc} in RML-infected murine brain homogenate using the aspartic endoprotease cathepsin D. This enzyme eliminates a short sequence at the C-terminal end of PrP to which the GPI anchor is attached. We have used this phenomenon as a tool to probe the impact of removal of the GPI anchor upon an *in vitro* amplification model of PrP^{Sc} [22], and have assayed prion infectivity using both a highly sensitive N2a-cell based bioassay, the scrapie cell assay [23], and a mouse bioassay [24]. We found that, in these systems, removal of the GPI anchor has no impact on either amplification of protease-resistant PrP or prion infectivity.

MATERIALS AND METHODS

Source of homogenates

Whole mouse brains from normal CD-1 mice and those inoculated with the RML strain of prion disease were homogenized as a 10% (w/v) preparation in Dulbecco's PBS without Ca²⁺ and Mg²⁺ ions using a Dounce homogenizer. Homogenates were then divided into 100 µl aliquots and were stored at -80 °C.

Protease treatment

Digests were carried out using cathepsin D purified from bovine spleen (Merck), freshly made up in 1 × PBS. Standard digest conditions were as follows: 10% infectious brain homogenates were thawed and centrifuged at 100 g for 1 min. Aliquots (10 µl) of 10% homogenate supernatant were then digested with 100 units of cathepsin D for 4 h at 37 °C with shaking at 450 rev./min. Following this, samples were digested with PK (proteinase K) (Sigma) at a final concentration of 50 µg/ml for 1 h at 37 °C. Digests were terminated by the addition of 2 × SDS sample buffer [125 mM Tris/HCl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 4% (v/v) 2-mercaptoethanol, 0.02% (w/v) Bromophenol Blue] containing 8 mM AEBF [4-(2-aminoethyl)benzenesulphonyl fluoride] (Pefabloc SC, Roche). In the case of cathepsin D digests carried out in the presence of EDTA, 20 mM EDTA (Sigma) was added to the reaction mixture and RML control samples.

Western blot analyses

Following the addition of 20 µl of 2 × SDS loading buffer, samples were heated to 100 °C for 10 min and then subjected to centrifugation in a microfuge (15 000 g) for 1 min. Each supernatant (20 µl) was applied to a 16% Tris/glycine gel (Novex; Life Technologies) according to the manufacturer's instructions. Gels were electroblotted on to PVDF membrane (Immobilon-P; Millipore) and were subsequently blocked in PBST [PBS containing 0.05% (v/v) Tween 20] and 5% (w/v) non-fat dried milk powder for 60 min. After washing in PBST, the membranes were incubated

with anti-PrP monoclonal antibody ICSM35 (D-Gen Ltd) diluted to 0.2 µg/ml in PBST for at least 60 min before washing in PBST (30 min) and incubation with an alkaline-phosphatase-conjugated goat anti-mouse antibody (Sigma), diluted 1:10 000 in PBST for 60 min. Following washing in PBST (30 min), the membranes were developed using AttoPhos reagent (Promega) and were visualized on a Molecular Dynamics Storm 840 instrument (Amersham Biosciences).

Cell culture

The murine neuroblastoma cell line, N2a, was used throughout. Cells were cultured in OFCS [Opti-MEM[®] (Invitrogen) with 10% foetal calf serum] and 100 units of both penicillin and streptomycin (Invitrogen)/ml. PI-PLC treatment of cells was carried out by removal of growth medium from confluent cells in a 10-cm-diameter dish and addition of PBS containing either 200 m-units of PI-PLC (Sigma) or 100 units of cathepsin D. Cells were incubated for 3 h, and the culture supernatant was harvested. Culture supernatant (300 µl) was mixed with 4 vol. of ice-cold methanol and centrifuged at 25 000 g for 30 min at 4 °C. The precipitated protein was then resuspended in 20 µl of PBS and analysed by Western blot as described above.

In vitro amplification

Analysis of *in vitro* amplification of PrP^{Sc} was carried out using a modification of the protocol from Lucassen et al. [22]. Brain homogenates (10%, w/v) were produced in PBS from RML-infected CD-1, wild-type CD-1 and PrP-null (FVB/N *Prnp*^{0/0}) mouse brains. RML homogenates were produced in the presence of 1% (v/v) Triton X-100 (Sigma), with the wild-type and PrP-null homogenates containing 1 × Complete[™] protease inhibitors (Roche). RML homogenate (20 µl) was digested with 200 units of cathepsin D for 2 h and then, in parallel with undigested RML control, diluted 1:25 into 1 × PBS with 1% (v/v) Triton X-100. These were then diluted 1:1 with CD-1 wild-type or PrP-null brain homogenate and incubated for 16 h at 37 °C with shaking at 450 rev./min. Following incubation, homogenates were digested with PK at a final concentration of 50 µg/ml for 1 h at 37 °C and were analysed by Western blotting. Each condition was repeated in triplicate.

Scrapie cell assay

High-sensitivity cell culture assays for prion infectivity were carried out as described by Klöhn et al. [23]. Briefly, PK1 cells, a highly scrapie-susceptible N2a subclone, were exposed for 3 days in 96-well plates to serial dilutions (10⁻⁴, 10⁻⁵, 10⁻⁶ and 10⁻⁷) of infectious RML homogenate either treated with cathepsin D or untreated as a control. The cells were then grown to 80% confluence and were split twice before finally being grown to confluence and resuspended in OFCS. Cells at 25 000/well were transferred to an ELISPOT (enzyme-linked immunospot plate) (Millipore). The cells were then treated for 1 h with 0.5 µg/ml PK in lysis buffer [50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.5% (w/v) sodium deoxycholate and 0.5% (v/v) Triton X-100], washed and denatured by treatment with 3 M guanidine isothiocyanate (Sigma). Wells were then washed and blocked before probing with anti-PrP monoclonal antibody ICSM18 (D-Gen Ltd), washing with TBST [Tris-buffered saline with 0.05% (v/v) Tween 20] and probing with alkaline-phosphatase-conjugated anti-IgG1. Scrapie-positive cells were identified after treatment with alkaline-phosphatase-conjugated substrate (Bio-Rad) using the Zeiss KS ELISPOT system.

Figure 1 Cathepsin D digestion of PrP^{Sc}

Mouse RML, hamster 263K and human types 1, 2, 3 and 4 PrP^{Sc} visualized by Western blot (A). Samples were digested with 20 units of cathepsin D per μ l of 10 % (w/v) brain homogenate and exhibit retarded migration in SDS/PAGE as compared with untreated controls, indicative of C-terminal truncation and loss of GPI. Concentration- (B) and time- (C) dependence of gel retardation following digestion with cathepsin D (CatD) are also shown, exemplifying the enzymatic nature of the cleavage. Molecular-mass sizes are given in kDa; U, units.

Mouse bio-assay of cathepsin-D-treated PrP^{Sc}

Cathepsin-D-treated RML was bioassayed using Tg20 transgenic mice, which overexpress the murine *Prn-p* gene [24]. RML-infected brain homogenate (10 %) was treated with 20 units/ μ l cathepsin D for 2 h at 37 °C or was mixed with PBS and then diluted with PBS to 0.1 % final concentration. RML, cathepsin-D-treated RML, PBS- and cathepsin-D-only controls were then inoculated intracerebrally into anaesthetized Tg20 mice [24]. Animal care adhered to institutional guidelines, and mice were examined daily for clinical signs of prion disease. Brain samples were taken from all groups following death, analysed by Western blot for the presence of PrP^{Sc} and for neuropathological evidence of prion disease.

RESULTS

Cathepsin D digest of PrP^{Sc} strains

Treatment of 10 % brain homogenate from RML-infected mice with cathepsin D resulted in the PK-resistant PrP in these homogenates running at an apparently higher molecular mass than those in control homogenates as analysed by SDS/PAGE (Figure 1A). While proteolytic digestion should decrease RMM (relative molecular mass) owing to the loss of polypeptide material, it has been shown previously that apparent increases in RMM occur upon removal of GPI anchors by the enzyme PI-PLC. This effect is caused by the disproportionate amount of SDS that associates with the acyl chains of the GPI anchor on treatment compared with the polypeptide component [14,15]. Hence, removal of the anchor results in a significant loss of SDS-associated charge and a slower migration in the electric field.

To discover whether this was a prion-strain-specific phenomenon, PrP^{Sc} in brain homogenates isolated from hamster (strain 263K) and human (molecular strain types 1, 2 and 4 [25,26]) were subjected to proteolysis by cathepsin D. All exhibited the same apparent increase in RMM (Figure 1A). Using murine RML prions as a model system, the concentration- and time-dependence of this effect was examined (Figures 1B and 1C), with the apparent

Figure 2 Release of PrP from cell membranes

Release of PrP from wild-type N2a cells by PI-PLC (A) and cathepsin D (B). Cells were treated with the indicated concentration of enzyme for 3 h, and the supernatants were collected and analysed by Western blotting. The presence of PrP^C in the supernatant, recognized by the monoclonal antibody ICSM35, signifies release of the protein from the cell membrane upon either GPI cleavage (PI-PLC) or C-terminal truncation (cathepsin D; CatD). Molecular-mass sizes are given in kDa.

Figure 3 Impact of EDTA on cathepsin D digestion

Digestion of RML (A) and the effect of increasing Cu²⁺ on RML strain profile (B). The presence of the metal chelator EDTA had no effect on cathepsin D (CatD) digestion of RML, neither did the presence of Cu²⁺ (as CuSO₄) alter the apparent RMM of RML, indicating that the gel retardation following cathepsin D treatment is not metal-ion-dependent. Molecular-mass sizes are given in kDa.

RMM increase showing dependence upon both digestion time and concentration of cathepsin D.

Cathepsin-D-mediated release of PrP from intact cell membranes

If treatment with cathepsin D removes the GPI anchor, it would be expected that treatment of intact cells in culture with the enzyme would result in release of membrane-anchored PrP^C into the cell medium. To examine this, wild-type N2a cells were treated with either cathepsin D or PI-PLC, with the incubation medium analysed for the presence of released PrP^C. Under these conditions, treatment with both cathepsin D and PI-PLC resulted in the release of PrP^C, as measured by an increase in the PrP^C collected from the medium (Figure 2). The quantity of PrP^C released from the surface of N2a cells appears to be less than that released by PI-PLC. This may be due, at least in part, to the degradation of released PrP^C by the high concentrations of cathepsin D in the reaction.

Change in strain profile is independent of metal ion binding

An alternative explanation for this alteration in electrophoretic properties following digestion with cathepsin D is that there is a change in conformation of the PrP^{Sc} that is dependent upon the inadvertent introduction of metal ions during the experimental process [27]. To eliminate this possibility, cathepsin D digests were carried out in the presence or absence of the metal-chelating agent EDTA, and the effects of increasing concentrations of Cu²⁺ upon the strain profile of RML were investigated (Figure 3A). The presence of EDTA did not affect the retardation of PrP

Figure 4 *In vitro* amplification of cathepsin-D-treated (Cat D +) and normal (Cat D –) RML

Treated or untreated RML-infected brain homogenate diluted into wild-type CD-1 brain homogenate both exhibited amplification of protease-resistant signal (compared with RML diluted into PrP^{0/0} brain homogenate) as quantified by band intensity following Western blot analysis. Results are means $\pm$ S.D. for three separate reactions. No significant difference was observed between treated and untreated samples.

in SDS/PAGE following cathepsin D digestion, neither did the addition of Cu²⁺ to RML result in any prion-strain-specific alteration in electrophoretic mobility (Figure 3B).

Loss of GPI anchor has no effect on *in vitro* amplification of PrP^{Sc}

To investigate the impact that GPI anchor loss has on the ability of PrP^{Sc} to replicate, cathepsin-D-treated RML was examined for its ability to seed amplification in an *in vitro* PrP^{Sc} replication model. Using the technique of Lucassen et al. [22], cathepsin-D-digested RML exhibited no significant difference in its ability to produce an amplified signal as compared with untreated RML (Figure 4).

Loss of GPI anchor has no effect on prion infectivity measured by scrapie cell assay

To determine whether the loss of its GPI anchor alters prion infectivity, cathepsin-D-treated RML prions were assayed for infectivity using a cell culture assay based upon a highly susceptible line of N2a cells, the scrapie cell assay. Using a range of dilutions, cathepsin-D-treated RML prion-infected brain homogenate was compared with untreated RML homogenate, along with cathepsin-D-only controls and CD-1 homogenate digested with cathepsin D (no cellular toxicity was observed with control treatments; results not shown). As RML alone exhibits a slight decrease in infectious titre upon incubation at 37°C, cathepsin-D-treated homogenates were compared with RML either added to the assay immediately following thawing or incubated in parallel with the cathepsin D digest. There was no significant reduction in prion titre following RML digestion by cathepsin D (Figure 5).

Loss of GPI anchor has no effect on prion infectivity measured using a mouse bioassay

Cathepsin-D-treated RML was then compared with untreated RML by bioassay in Tg20 transgenic mice, which overexpress mouse PrP^C [24]. Mice were inoculated with untreated RML, cathepsin-D-treated RML, PBS control, or with cathepsin D alone. Mice inoculated with cathepsin-D-treated RML and with untreated RML developed clinical scrapie after 9 weeks, with no significant difference between the incubation periods (Figure 6A).

Figure 5 Scrapie cell assay analysis of RML infectivity

RML was incubated for 4 h at 37°C or RML digested with cathepsin D (Cat D) for 4 h at 37°C. A range of dilutions of RML brain homogenates from 10^{−4} to 10^{−7} were used to allow estimation of any impact of cathepsin D digestion on the infectivity of RML, with no significant difference between digested and undigested samples. Results are means $\pm$ S.D. of the number of cells that contain detectable PrP^{RES} (PK-resistant PrP) per 25 000 viable cells for three independent experiments. UI, uninfected.

Figure 6 *In vivo* bioassay for infectivity in cathepsin-D-treated RML

Incubation times and attack rates of RML (A) and the presence of protease-resistant material in the brains of mice inoculated with both RML and cathepsin-D-treated RML (B). Tg20 transgenic mice were inoculated intracerebrally with 0.1% (w/v) RML brain homogenate and monitored for clinical signs of prion disease. Groups of mice inoculated with either cathepsin-D (Cat D)-treated RML or native RML exhibited similar incubation times shown in days post-inoculation. The presence of protease-resistant material in the brains of both experimental groups was confirmed by Western blotting. Control groups (PBS and cathepsin D alone) showed no evidence of prion disease. Molecular-mass sizes are indicated in kDa. SE, S.E.M.

Homogenates from both groups were examined for protease-resistant PrP, and were positive in both cases (Figure 6B). Mice were also examined for neuropathological hallmarks of scrapie infection, and both groups showed classical signs of prion disease.

DISCUSSION

Despite decades of research, both the precise molecular mechanism of pathological injury and the nature of the infectious agent in the prion disorders remains elusive. One of the areas of prion disease biology that is not clearly understood is the role of the post-translational modifications of PrP. The presence of altered glycoform ratios in different prion strains suggests that glycosylation may play a role in the propagation of strain properties [25,28], and studies using PI-PLC to remove the GPI anchor from PrP in infected cells indicate that perhaps this also plays a role in cell-to-cell propagation of infection [29]. A critical role for the GPI anchor in the development of prion disease has recently been demonstrated by Chesebro et al. [19], where cells lacking GPI-anchored PrP^C appear largely immune to the cytotoxic effects of PrP^{Sc}.

In this study, cathepsin D has been used to remove a short C-terminal sequence from infectivity-associated PrP^{Sc}. In doing so, three major technical difficulties prevented the precise characterization of the nature and location of the cleavage event: first, PrP^{Sc} is difficult to purify without denaturation; secondly, its hydrophobicity prevents direct sequencing of the cleavage products [26]; and thirdly, cathepsin D has a poorly defined cleavage consensus sequence (characterized only as a preference to cleave between two hydrophobic residues) [30,31]. However, the evidence of retarded migration of PrP in SDS/PAGE and the release of PrP from the cell membrane following digestion with cathepsin D indicate that cathepsin D removes a segment of the C-terminus of the protein and, with those amino acids, the GPI anchor. This provided an alternative approach to the use of PI-PLC treatment, and therefore denaturing conditions, as a means of evaluating the role of the GPI anchor in the infective process.

It should be noted that the concentrations of cathepsin D used to digest PrP^{Sc} were greatly in excess of physiologically relevant levels. It is intriguing, however, that cathepsin D has been implicated in the proteolytic processing of amyloid β -peptide, α -synuclein and Tau [32–34]. Cathepsin D is also up-regulated both in mouse models of scrapie [35,36] and in the brains of Alzheimer's disease patients (although this may reflect a general up-regulation of lysosomal degradation in these disorders, rather than a substrate-specific effect) [37]. A polymorphism in the human cathepsin D gene has also been identified as a risk factor for late-onset Alzheimer's disease, providing a potential link with other neurodegenerative protein-folding diseases [38].

The impact of the removal of the GPI anchor from infective PrP^{Sc} was studied in three systems: a model *in vitro* amplification system, the *ex vivo* scrapie cell prion assay and a mouse bioassay. No significant effect was observed with any of these techniques. The relationship of the first system to the *in vivo* situation remains a subject of debate. Several systems for the *in vitro* amplification of PrP^{Sc} have been reported to generate an amplification of signal, but no categorical proof of a concomitant increase in infectivity has been produced, and so these systems may reflect an increase in protease-resistant PrP rather than amplification of infectivity [39–42]. However, data from the *in vivo* assay of cathepsin-D-treated RML agree with those both from the *in vitro* amplification and from the highly sensitive cell culture analysis.

The evidence presented from these three systems demonstrates that the removal of the GPI anchor from PrP^{Sc} does not affect

the infective properties of the scrapie agent. This is particularly well demonstrated by the scrapie cell assay, which is capable of detecting just a 2-fold change in infective titre. One constraint on the correlation of Western blot detection of PrP and infectivity is the limited dynamic range of immunodetection. It can be determined by Western blotting that 99 % of the detectable material has been cleaved by cathepsin D (Figure 1), but this is an insignificant amount compared with the very high titres of infectivity in the starting homogenate ($> 10^7$ LD₅₀ units). Although a 100-fold change in the titre of infectivity can be detected in a conventional mouse bioassay, this would result in a marginal increase in incubation time. We see no evidence for any change in titre using mouse bioassay (Figure 6), but, more significantly, no change in titre is detected using a cell culture assay that is capable of detecting changes in titre as small as 2-fold (Figure 5).

This finding has two major implications. First, since endogenous proteolytic release of PrP^C has been reported [43], this may provide a mechanism whereby infectious PrP^{Sc} could spread from cell to cell and throughout the body of an infected host. Secondly, the fact that prion infectivity does not require a GPI anchor means that this is not a factor preventing the production of *de novo* prion infectivity using recombinant protein from bacterial sources. This is in agreement with the recent demonstration by Legname et al. [21] that PrP generated in *Escherichia coli* cells by recombinant expression is capable of inducing prion disease in transgenic mice with high levels of overexpression of a truncated PrP.

This work was funded by the Medical Research Council. We are grateful to Ray Young for his assistance in preparation of the Figures for this manuscript.

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Received 18 October 2005/9 January 2006; accepted 27 January 2006

Published as BJ Immediate Publication 27 January 2006, doi:10.1042/BJ20051677