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Detection and characterization of proteinase K-sensitive disease-related prion protein with thermolysin

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Disease-related PrP^{Sc} [pathogenic PrP (prion protein)] is classically distinguished from its normal cellular precursor, PrP^{C}(cellular PrP) by its detergent insolubility and partial resistance to proteolysis. Although molecular diagnosis of prion disease has historically relied upon detection of protease-resistant fragments of PrP^{Sc} using PK (proteinase K), it is now apparent that a substantial fraction of disease-related PrP is destroyed by this protease. Recently, thermolysin has been identified as a complementary tool to PK, permitting isolation of PrP^{Sc} in its full-length form. In the present study, we show that thermolysin can degrade PrP^{C} while preserving both PK-sensitive and PK-resistant isoforms of disease-related PrP in both rodent and human prion strains. For mouse RML (Rocky Mountain Laboratory) prions, the majority of PK-sensitive disease-related PrP isoforms do not appear to contribute significantly to infectivity. In vCJD (variant Creutzfeldt–Jakob disease), the human counterpart of BSE (bovine spongiform encephalopathy), up to 90 % of total PrP present in the brain resists degradation with thermolysin, whereas only ~15 % of this material resists digestion by PK. Detection of PK-sensitive isoforms of disease-related PrP using thermolysin should be useful for improving diagnostic sensitivity in human prion diseases.

Key words: prion, prion protein (PrP), scrapie, thermolysin, transmissible spongiform encephalopathy, variant Creutzfeldt–Jakob disease (vCJD).

INTRODUCTION

Prion diseases are fatal neurodegenerative disorders that include CJD (Creutzfeldt–Jakob disease), GSS (Gerstmann–Sträussler–Scheinker disease), FFI (fatal familial insomnia), kuru and variant CJD (vCJD) in humans [1–3]. Their central feature is the post-translational conversion of host-encoded PrP^{C} [cellular PrP (prion protein)], into an abnormal isoform, designated PrP^{Sc} (pathogenic PrP) [1,2]. Human prion diseases are biologically unique in that the disease process can be triggered through inherited germline mutations in the human PrP gene (*PRNP*), infection (by inoculation, or in some cases by dietary exposure) with prion-infected tissue, or by rare sporadic events that generate PrP^{Sc} [1–4]. According to the protein-only hypothesis [5], an abnormal PrP isoform is the principal, if not the sole, component of the transmissible prion, with prion propagation occurring through PrP^{Sc} acting to replicate itself with high fidelity by recruiting endogenous PrP^{C} [1,2,6,7]. Within the framework of the protein-only hypothesis of prion propagation, the distinct clinical and neuropathological phenotypes that distinguish prion strains are thought to be determined by the propagation of PrP^{Sc} isoforms with divergent physicochemical properties [1,2,7–12].

PrP^{Sc} is extracted from affected tissue as highly aggregated detergent-insoluble material that is not amenable to high-resolution structural techniques. However, FTIR (Fourier-transform infrared) spectroscopic methods show that PrP^{Sc}, in distinction from PrP^{C}, has a high β-sheet content [13,14]. Biochemically, PrP^{Sc} can be distinguished from PrP^{C} by its partial resistance to proteolysis and its marked insolubility in detergents (for reviews see [1,15]). Under conditions in which PrP^{C} exists as a detergent-soluble monomer and is completely degraded by the non-specific protease PK (proteinase K), PrP^{Sc} exists in an aggregated form with the C-terminal two-thirds of the protein showing a marked resistance to proteolytic degradation, leading to the generation of N-terminally truncated fragments of di-, mono- and non-glycosylated PrP [1,15].

Although the molecular diagnosis of prion disease has historically relied upon the detection of PrP^{Sc} using PK, it has become apparent that PK-sensitive pathological isoforms of PrP may have a significant role in prion disease pathogenesis [12,16–21]. In particular, in inherited prion disease, PrP^{Sc} isoforms may be generated with unique physicochemical properties, reflected by sensitivity to PK digestion and PrP^{Sc}/prion infectivity ratios that can be very different from the PrP^{Sc} types propagated in sporadic and acquired forms of human prion disease (for reviews see [2,3,22]). Accordingly, the development of new diagnostic tests that do not rely on PK digestion is required, and, in this context, a conformation-dependent immunoassay [12] shows high diagnostic sensitivity in human prion disease [18]. More recently, Gough and colleagues reported thermolysin as a complementary tool to PK. Thermolysin destroys PrP^{C} in ovine or bovine brain while leaving PrP^{Sc} in its full-length form, thereby allowing the N-terminal domain of PrP^{Sc} to be exploited for improved methods of prion-disease diagnosis [23] or prion-strain discrimination [24]. In the present study, we now extend these findings and show that thermolysin preserves both PK-sensitive and PK-resistant disease-related isoforms of PrP while concomitantly destroying PrP^{C} in both rodent and human brain. Using mouse RML

Abbreviations used: CJD, Creutzfeldt–Jakob disease; DPBS, Dulbecco's PBS; NaPTA, sodium phosphotungstic acid; PBST, PBS containing 0.05 % Tween 20; PK, proteinase K; PNGase F, peptide N-glycosidase F; PrP, prion protein; PrP^{C}, cellular PrP; PrP^{Sc}, pathogenic PrP; RML, Rocky Mountain Laboratory; vCJD, variant CJD.

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(Rocky Mountain Laboratory) prions, we were able to investigate prion infectivity associated with PK-sensitive PrP isoforms. It is anticipated that these methods will facilitate detailed biochemical characterization of PK-sensitive isoforms of disease-related PrP associated with multiple prion strain/host combinations.

MATERIALS AND METHODS

Prion-infected tissues

Storage and biochemical analysis of human brain samples was performed with consent from relatives and with approval from the Local Research Ethics Committee of the Institute of Neurology/National Hospital for Neurology and Neurosurgery (London, U.K.). All procedures were carried out in a microbiological containment level III facility with strict adherence to safety protocols. Brain homogenates [10 % (w/v)] from patients with neuropathologically confirmed vCJD or from control samples of normal human brain were prepared in DPBS (Dulbecco's PBS) lacking Ca^{2+} or Mg^{2+} ions by serial passage through needles of decreasing diameter or the use of tissue grinders (Anachem) [10,25]. Brains from 200 terminal CD-1 mice infected with the RML prion strain [26] were homogenized in DPBS lacking Ca^{2+} or Mg^{2+} ions using tissue grinders and pooled to produce 970 ml of 10 % (w/v) RML brain homogenate (designated I6200). Two batches of 18 brains from uninfected CD-1 mice were homogenized to produce two pools of ~90 ml of 10 % (w/v) normal CD-1 brain homogenate (designated I7219 and I8402). Similar procedures were used to generate 10 % (w/v) brain homogenates from hamsters terminally infected with the Sc237 prion strain or from normal uninfected hamsters. Pooled homogenates were dispensed as aliquots and maintained at −70 °C until use.

Titration of RML prions in CD-1 mice

All procedures were carried out in a microbiological containment level III facility with strict adherence to safety protocols. Care of mice was performed according to institutional and national animal care committee guidelines. RML brain homogenate (I6200) [10 % (w/v)] was serially diluted (10^{-1} to 10^{-8}) using 1 % (w/v) normal CD-1 brain homogenate as diluent. Aliquots of each dilution were inoculated either intracerebrally (30 μl) or intraperitoneally (100 μl) into groups of 6 CD-1 mice as described previously [27–29]. Mice were examined daily and were killed if exhibiting signs of distress or once a diagnosis of clinical prion disease was established [30]. Infectious prion titre (LD_{50}) was calculated using the Reed–Muench formula [31].

Enzymatic digestion

Thermolysin (EC 3.4.24.27) from *Bacillus thermoproteolyticus rokko* was obtained freeze-dried from Sigma–Aldrich. The specific enzymatic activity is 50–100 units/mg of protein (where 1 unit liberates 1 μmol of tyrosine/min at pH 7.5 and 37 °C using casein as a substrate). PK (EC 3.4.21.64) from *Tritirachium album limber* was obtained freeze-dried from Merck. The specific enzymatic activity is approx. 30 Anson units/g (where 1 Anson unit is the amount of enzyme that liberates 1 mmol of Folin-positive amino acids/min at pH 7.5 and 35 °C using haemoglobin as a substrate). Stock solutions of 1 mg/ml thermolysin or PK were prepared in water and aliquots were stored at −70 °C. Aliquots of 10 % (w/v) brain homogenates in DPBS were digested for variable time periods with thermolysin at a final protease concentration of 100 μg/ml at 70 °C or 37 °C, or with PK at a final concentration of 50 μg/ml (mouse brain) or 100 μg/ml (human brain) at 37 °C. Aliquots of the digests were snap-frozen for infectivity studies, or processed immediately for analysis by either immunoblotting or ELISA. Enzymatic deglycosylation of PrP prior to immunoblotting was accomplished by incubating 20 μl aliquots of 2% (w/v) SDS and heat-denatured brain homogenate with 1000 units of recombinant PNGase F (peptide N-glycosidase F) (New England Biolabs) in buffer containing 1 % Nonidet P40 for 2 h at 37 °C according to the manufacturer's instructions. Samples were precipitated with 100 % acetone for 1 h at −20 °C and centrifuged at 16 100 ***g*** for 30 min in a microfuge. Pellets were resuspended in 1× SDS sample buffer and analysed by immunoblotting.

PrP immunoblotting

Aliquots of brain homogenate were mixed with an equal volume 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 and 0.02 % Bromophenol Blue containing 8 mM 4-(2-aminoethyl)-benzenesulphonyl fluoride] and immediately transferred to a 100 °C heating block for 10 min. Samples were analysed by electrophoresis (16 % gels) and immunoblotting as described previously [22,25]. Blots were blocked in PBST [PBS containing 0.05 % Tween 20] and 5 % (w/v) non-fat dried skimmed milk powder and probed with the anti-PrP monoclonal antibodies ICSM 35 (D-Gen), 3F4 (Signet Laboratories) or SAF32 (Spibio) at 0.2 μg/ml final antibody concentration in PBST. Blots were developed using alkaline-phosphatase-conjugated anti-mouse IgG secondary antibody and chemiluminescent substrate CDP-Star (Tropix), and visualized on Biomax MR film (Kodak) as described previously [25]. Densitometric analysis of PrP was performed by using Scion Image analysis software (Scion Corporation).

Detergent-solubility studies

Aliquots of 10 % (w/v) brain homogenate (20 μl) were treated with 0.5 μl of benzonase (Benzon nuclease, purity 1; Merck). Samples were subsequently adjusted with 80 μl of PBS and 100 μl of PBS containing 4 % (w/v) sodium lauroylsarcosine (Calbiochem) and incubated for 30 min at 37 °C with constant agitation. Samples were then centrifuged at 16 100 ***g*** for 30 min in a microfuge to generate soluble (supernatant) or insoluble (pellet) fractions. Soluble protein in the supernatant was precipitated with 1 ml of cold methanol (−20 °C) and recovered by centrifugation at 16 100 *g* for 30 min in a microfuge. The original detergent-insoluble pellets and methanol-precipitated supernatant protein pellets were re-suspended to a final volume of 40 μl with PBS containing 0.1 % (w/v) sodium lauroylsarcosine, and 10 μl aliquots were either left untreated or digested with thermolysin (100 μg/ml final protease concentration) at 70 °C for 30 min or PK (50 μg/ml final protease concentration) at 37 °C for 1 h. Samples were analysed by electrophoresis and immunoblotting as described above.

ELISA detection of PrP

ELISA was performed using methods described previously [32] with adaptations. Brain homogenates were treated with thermolysin (100 μg/ml final protease concentration) at 70 °C or 37 °C or PK (50 or 100 μg/ml final protease concentration) at 37 °C for a range of incubation times. Subsequently, 10 μl aliquots of these samples or untreated brain homogenate and temperature controls were adjusted with 10 μl of 4 % (w/v) SDS and heated at 100 °C for 10 min. Samples were centrifuged at 100 ***g*** for 30 s before adjustment with 600 μl of 50 mM Tris/HCl (pH 8.4) containing 2 % (v/v) Triton X-100, 2 % (w/v) sodium lauroylsarcosine and 2 % (w/v) bovine serum albumin

(Fraction V, protease free, Sigma–Aldrich). Aliquots (50 μl) were transferred into the wells of microtitre plates (Microlon 96W, Greiner Bio-One) containing immobilized anti-PrP monoclonal antibody ICSM18 (250 ng/well; D-Gen). After incubation at 37 °C for 1 h with constant agitation, wells were washed with 3 × 300 μl of PBST using an automated microplate washer, followed by the addition of 100 μl of PBS containing 1 % Tween 20 and 1 μg/ml biotinylated anti-PrP monoclonal antibody ICSM35 (D-Gen). Following incubation at 37 °C for 1 h with constant agitation, wells were washed as detailed above, followed by the addition of 100 μl of PBS containing 1 % Tween 20 and a dilution of streptavidin–horseradish-peroxidase conjugate (1:10 000 dilution, Dako). After incubation at 37 °C for 30 min with constant agitation, wells were washed with 4 × 300 μl of PBST. Wells were developed with 100 μl of QuantaBlu working solution (Pierce) and the reactions were stopped by the addition of 100 μl of QuantaBlu stop solution (Pierce). Fluorescence ($\lambda_{ex} = 325$ nm, $\lambda_{em} = 425$ nm) was measured on a Tecan spectra image microplate reader.

NaPTA (sodium phosphotungstic acid) precipitation

The use of NaPTA was performed using a protocol adapted from Safar et al. [12] essentially as described previously [25]. Briefly, 100 μl aliquots of 10 % (w/v) brain homogenate were treated with 1 μl of benzonase and were subsequently either digested with thermolysin (100 μg/ml final protease concentration) at 37 °C for 90 min or left untreated. Thermolysin digestion was stopped by the addition of EDTA (10 mM final concentration). Samples were subsequently adjusted with 100 μl of PBS containing 4 % (w/v) sodium lauroylsarcosine, incubated at 37 °C for 30 min with constant agitation, and then further adjusted with 16.3 μl of a stock solution containing 4 % (w/v) NaPTA (lacking magnesium chloride) (pH 7.4) to give a final concentration in the sample of 0.3 % (w/v). Samples were incubated at 37 °C for 30 min with constant agitation before centrifugation at 16 100 ***g*** for 30 min in a microfuge. After careful isolation of the supernatant, the pellets were resuspended to 100 μl with PBS. Aliquots were processed immediately for analysis by ELISA.

Scrapie cell assay

High-sensitivity cell-culture assays for RML prion infectivity were performed as described previously by Klohn et al. [33]. Briefly, PK1 cells, a highly scrapie-susceptible N2a subclone, were exposed for 3 days in 96-well plates to serial dilutions (10^{-3} and 10^{-4}) of 10 % (w/v) RML brain homogenate either untreated or following digestion with thermolysin (100 μg/ml final protease concentration) at 37 °C or 70 °C or PK (50 μg/ml final protease concentration) at 37 °C for a range of incubation times. A serial dilution of untreated 10 % (w/v) RML brain homogenate (3×10^{-4} to 3×10^{-6}) of known infectivity titre was performed in parallel. Subsequently cells were split and passaged appropriately for the scrapie cell assay as described previously [33] and the infectivity titre of each sample was deduced from the reference preparation.

Statistical analysis

All experiments were conducted at least three times. Figures show representative data and show means ± S.E.M. or S.D.

RESULTS

Characterization of RML prion-infected brain homogenate

In order to standardize a large series of experiments, we generated a 970 ml stock of 10 % (w/v) RML prion-infected brain homogenate from terminally affected CD-1 mice and titrated this by intracerebral or intraperitoneal inoculation in CD-1 mice (Table 1). Using the Reed–Muench formula, 10 % (w/v) RML brain homogenate (I6200) has an infectious prion titre of $10^{8.3}$ intracerebral LD_{50}/ml and $10^{6.6}$ intraperitoneal LD_{50}/ml. Examination of 10 % (w/v) RML brain homogenate by immunoblotting before or after standard PK digestion (50 μg/ml PK for 1 h at 37 °C) showed the expected pattern of PrP bands (Figure 1a). In the absence of protease digestion, a mixture of full-length and truncated PrP species is observed. After PK digestion, the characteristic pattern of N-terminally truncated fragments of di-, mono- and non-glycosylated PrP is observed, with a predominance of mono-glycosylated PrP (Figure 1a).

Table 1 Titration of RML prions in CD-1 mice

Each dilution of 10 % (w/v) RML brain homogenate I6200 was performed in 1 % normal CD-1 brain homogenate. Mice were inoculated intracerebrally (I.c.) with 30 μl of each dilution or intraperitoneally (I.p.) with 100 μl of each dilution, and the incubation periods are reported for clinically affected animals in days. Results are means ± S.D. ($n \geqslant 3$). n, number of affected mice; n_0, number of inoculated mice.

	I.c. inoculation		I.p. inoculation	
Dilution	n/n_0	Mean survival time (days)	n/n_0	Mean survival time (days)
10^{-1}	6/6	134 ± 2	6/6	182 ± 12
10^{-2}	5/5	152 ± 7	6/6	184 ± 13
10^{-3}	6/6	154 ± 3	6/6	200 ± 5
10^{-4}	6/6	162 ± 12	6/6	203 ± 8
10^{-5}	6/6	167 ± 4	6/6	209 ± 7
10^{-6}	4/4	211 ± 17	1/4	208
10^{-7}	2/6	208, 222	0/6	–
10^{-8}	1/6	287	0/6	–

Thermolysin degrades mouse PrP^C while preserving full-length PrP^{Sc}

In initial experiments we adapted the methods of Owen et al. [23] to determine the conditions in which thermolysin could efficiently degrade mouse PrP^C. We found that digestion of 10 % (w/v) normal CD-1 brain homogenate with 100 μg/ml thermolysin for 30 min at 70 °C efficiently degraded PrP^C (Figure 1b). In agreement with Owen et al. [23], silver-stain analysis of thermolysin-digested samples revealed that the majority of proteins in the brain homogenate were destroyed by this treatment (results not shown). In sharp contrast, application of the same thermolysin digestion conditions to 10 % (w/v) RML prion-infected brain homogenate produced no apparent change in the PrP fragment pattern and only a modest reduction in the overall signal intensity (Figure 1b). These results (which are in accordance with the findings of Owen et al. [23] using ovine or bovine brain homogenates) indicate that thermolysin can efficiently degrade mouse PrP^C while leaving PrP^{Sc} intact. The presence of full-length PrP in thermolysin-digested RML brain homogenate was confirmed through the use of an N-terminal-specific anti-PrP monoclonal antibody and by deglycosylation. The SAF32 monoclonal anti-PrP antibody, with an epitope spanning residues 79–92 of hamster PrP [34], detected all three full-length PrP glycoforms in thermolysin-digested samples (Figure 1d). After deglycosylation, thermolysin-resistant PrP migrated as two bands (Figure 1b) with apparent molecular masses corresponding to either full-length PrP or an endogenously truncated C2 fragment that has been characterized previously [24,35,36]. Densitometry showed that ~25 % of the total PrP remaining after 30 min digestion with thermolysin was in the full-length form. Importantly, short time periods of digestion

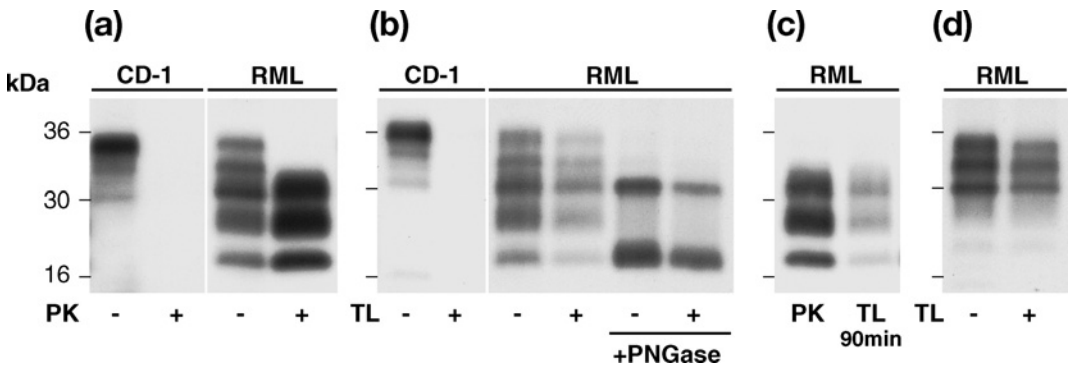

Figure 1 PK and thermolysin digestion of mouse PrP

Brain homogenate [10 % (w/v)] from uninfected CD-1 mice (CD-1) and RML prion-infected CD-1 mice (RML) were analysed by immunoblotting in the absence of protease digestion (−) or after digestion (+) with PK (50 μg/ml at 37 °C for 1 h) (**a**) or thermolysin (TL) (100 μg/ml at 70 °C) for 30 min (**b**, **d**) or 90 min (**c**). Non-glycosylated full-length and endogenously truncated PrP species were detected after deglycosylation with PNGase F (+PNGase). Immunoblots were probed with anti-PrP monoclonal antibodies ICSM35 (**a**–**c**) or SAF32 (**d**). Molecular masses are stated on the left-hand side (in kDa).

with 100 μg/ml thermolysin at 70 °C appeared to be required for isolation of full-length PrP^{Sc} from RML brain homogenate. Prolonged digestion (90 min) with thermolysin at 70 °C leads to the generation of truncated PrP, with a migration pattern equivalent to that observed after digestion with PK (Figure 1c). As thermolysin has no preferred cleavage sites in the N-terminal region of PrP [23], the generation of truncated PrP may be attributable to thermolysin acting at a non-preferred scissile bond or to the activity of a minor contaminating protease. Truncation of disease-related PrP by this activity appears to be prion-strain specific [24], and these results indicate that the conditions for digestion with thermolysin should be optimized empirically for different prion strain/host combinations.

Thermolysin-resistant PrP is mainly insoluble and comprises both PK-sensitive and PK-resistant PrP

In addition to protease resistance, disease-related isoforms of PrP are defined by their aggregation state and insolubility in non-denaturing detergents. Therefore we examined the detergent solubility of thermolysin-resistant PrP. Aliquots of brain homogenate were incubated with 2 % (w/v) sodium lauroylsarcosine and then centrifuged to generate detergent-soluble supernatants and detergent-insoluble pellets. Aliquots of these fractions were subsequently digested with thermolysin or PK or left untreated. Immunoblot analysis of PrP present in the supernatant and pellet fractions showed that the majority of thermolysin-resistant PrP from RML brain is insoluble (Figure 2a). However, under the same conditions, PrP^{C} from normal CD-1 brain was entirely soluble and was recovered only in the supernatant fraction (Figure 2b). These findings show that thermolysin-resistant PrP in RML brain homogenate has the properties of disease-related PrP.

Notably, we often observed an apparent increase in PrP signal strength on immunoblots after digestion with PK (e.g., Figure 1a). Therefore to assess reliably the proportions of total PrP in RML brain homogenate that are resistant to digestion with either thermolysin or PK, we used an ELISA protocol that provides a sensitive means for the immunodetection of denatured PrP regardless of whether this is derived from PrP^{C} or PrP^{Sc} ([32] and S. Cronier and M. H. Tattum, unpublished work). After 30 min of digestion of RML brain homogenate with 100 μg/ml thermolysin at 70 °C (conditions that leave PrP^{Sc} intact and efficiently degrade PrP^{C}), ∼55 % of total PrP was preserved (Figure 3). In contrast, after 30 min digestion with 50 μg/ml

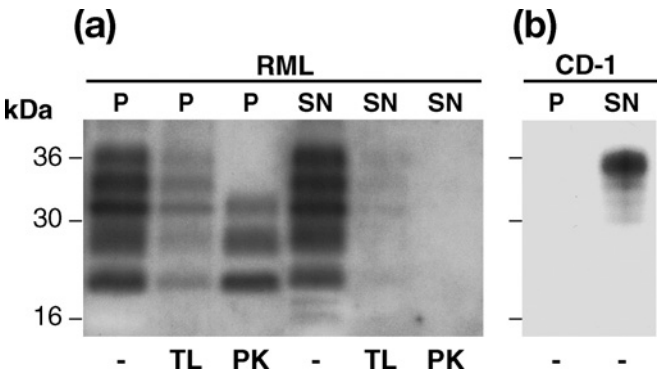

Figure 2 Thermolysin-resistant PrP from RML brain is mainly insoluble

Aliquots of 10 % (w/v) RML brain homogenate (**a**) or untreated 10 % (w/v) normal CD-1 brain homogenate (**b**) were diluted 10-fold in the presence of 2 % (w/v) sodium lauroylsarcosine and incubated for 30 min prior to centrifugation at 16 100 ***g*** for 30 min. PrP recovered in the detergent-insoluble pellets (P) or detergent-soluble supernatants (SN) was analysed before (−) or after digestion with thermolysin (TL) (100 μg/ml, 70 °C, 30 min) or PK (50 μg/ml, 37 °C, 1 h) by immunoblotting with the anti-PrP monoclonal antibody ICSM35. Molecular masses are stated on the left-hand side (in kDa).

PK at 37 °C, only ∼20 % of total PrP remained (Figure 3). After 5 min digestion with these protease concentrations at these temperatures, PrP^{C} was completely degraded in normal CD-1 brain homogenate (Figure 3). At this time point, in RML brain homogenate, there was an even higher differential between the PrP remaining after thermolysin (∼85 %) or PK (∼20 %) treatment. To investigate the reproducibility of this finding, instead of using pooled RML brain homogenate, we examined 10 % (w/v) brain homogenates prepared from four individual terminal RML-infected CD-1 mice. After 5 min digestion, the mean percentages of thermolysin-resistant PrP and PK-resistant PrP ($\pm$ S.D.) were highly reproducible, giving values of 91 $\pm$ 6 % and 23 $\pm$ 3 % respectively. Notably, prolonged digestion (90 min) with thermolysin leads to further degradation of disease-related PrP, reaching levels comparable with those seen after digestion with PK (Figure 3). These results can be correlated with the immunoblot analysis of thermolysin digests, which show a change from a mixture of full-length and truncated PrP species at 30 min to only truncated PrP after 90 min (compare Figures 1b and 1c). Thus the conditions defined here show that for this prion strain/host combination, disease-related PrP in RML brain

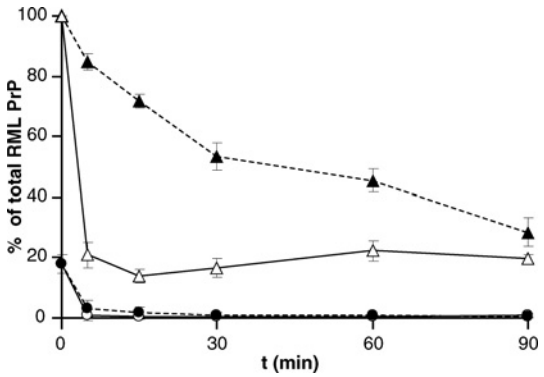

Figure 3 Measurement of PK-sensitive PrP in RML brain by ELISA

Normal CD-1 (●, ○) and RML (▲, △) brain homogenates [10 % (w/v)] were digested with thermolysin (100 μg/ml at 70 °C, ●, ▲; broken line) or with PK (50 μg/ml at 37 °C, ○, △; solid line) for a range of incubation times (t). PrP concentration was measured by ELISA in untreated normal CD-1 and RML brain homogenates and following protease digestion. PrP concentration is expressed as a percentage of total PrP present in untreated RML brain homogenate. Results are means ± S.E.M. ($n = 3$).

homogenate is degraded at different rates by thermolysin or PK, with the majority of disease-related thermolysin-resistant PrP being rapidly degraded by PK under standard digestion conditions.

The majority of PK-sensitive disease-related isoforms of PrP do not contribute to RML prion infectivity

Our results show that digestion of 10 % (w/v) RML-infected brain homogenate with 100 μg/ml thermolysin for 30 min at 70 °C leads to the efficient degradation of PrP^C while preserving both PK-resistant and PK-sensitive disease-related isoforms of PrP. To determine whether the PK-sensitive PrP species contribute to RML prion infectivity, we measured prion infectivity in PK- and thermolysin-digested brain homogenate using the scrapie cell assay [33] and compared this with the PrP content determined in parallel by ELISA and immunoblotting. As control experiments, we first investigated the effects of temperature alone on prion infectivity in non-digested RML brain homogenate. However, we found that incubation of brain homogenate at 70 °C rapidly destroyed up to 90 % of RML prion infectivity (Figure 4a). Clearly, exposure to 70 °C is incompatible with conducting meaningful RML prion infectivity studies, and this finding is in close agreement with the temperatures shown to produce substantial inactivation of other rodent-adapted prion strains [37]. Accordingly, we investigated the effect of incubating RML brain homogenate at 37 °C. At this temperature, a reproducible fluctuation in prion infectivity was observed over a 90 min time course (Figure 4b). Overall, however, the loss of infectivity at 37 °C was more modest and acceptable than seen at 70 °C, with ~65 % of starting infectivity remaining after 90 min of incubation (Figure 4b). Importantly, as shown in Figure 5, the majority of PrP^C from normal CD-1 brain was destroyed after 5 min incubation with 100 μg/ml thermolysin at 37 °C, with ~10 % PrP remaining after digestion for 90 min (also see Figure 6). In normal CD-1 brain homogenate, PrP^C level is around ~20 % of total PrP present in untreated RML brain homogenate (Figure 3, compare PrP content at 0 min). Therefore assuming that the PrP^C expression level is similar in uninfected and RML-infected CD-1 brains, PrP^C would correspond to only ~2 % of total PrP in RML brain homogenate after digestion with thermolysin for 90 min at 37 °C.

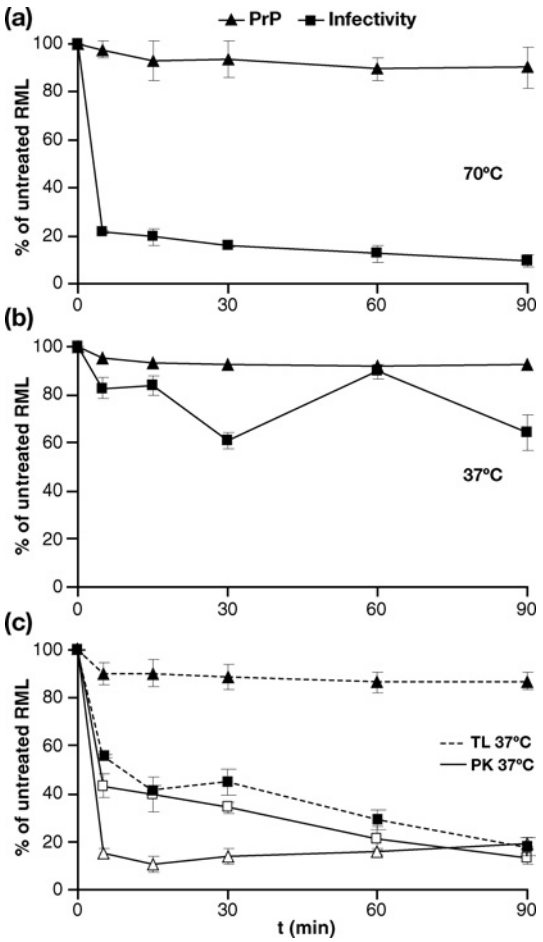

Figure 4 RML prion infectivity and PrP content following digestion with thermolysin or PK

For each sample, PrP (▲) was quantified by ELISA and expressed as a percentage of total PrP present in the untreated sample. RML prion infectivity (■) was measured by the scrapie cell assay and expressed as a percentage of total infectivity present in the untreated sample. (**a**, **b**) RML brain homogenate [10 % (w/v)] incubated without protease at either 70 °C (**a**) or 37 °C (**b**) for various incubation times (t). (**c**) RML brain homogenate [10 % (w/v)] incubated at 37 °C with either PK (50 μg/ml, □, △; solid line) or thermolysin (100 μg/ml, ■, ▲; broken line) at 37 °C for various incubation times (t). Results are means ± S.E.M. ($n = 3$).

On the basis of these results, we performed comparative experiments on RML brain homogenate with thermolysin and PK at 37 °C. After digestion with thermolysin for 90 min, ~85 % of total PrP was preserved (Figure 4c and Figure 6). In sharp contrast, only ~20 % of total PrP was preserved in RML brain homogenate after the equivalent digestion with PK at 37 °C (Figure 4c). All thermolysin-resistant PrP in RML brain homogenate (comprising both the full-length and truncated PrP isoforms) was precipitated by NaPTA, whereas no PrP^C from thermolysin-digested normal CD-1 brain was recovered by this treatment (Figure 6). These results show that disease-related PrP comprises up to 85 % of total PrP present in RML mouse brain homogenate; however, the majority (~75 %) of these disease-related PrP isoforms are readily sensitive to rapid digestion by PK. This finding agrees well with previous studies of RML-infected mouse brain, which also showed that only a minority of total disease-related PrP is PK-resistant [38,39]. Strikingly, contrary to the marked disparity in PrP content, infectivity titres in both PK- and thermolysin-treated samples remained closely similar throughout the time course of digestion (Figure 4c). After 90 min digestion, ~20 % of

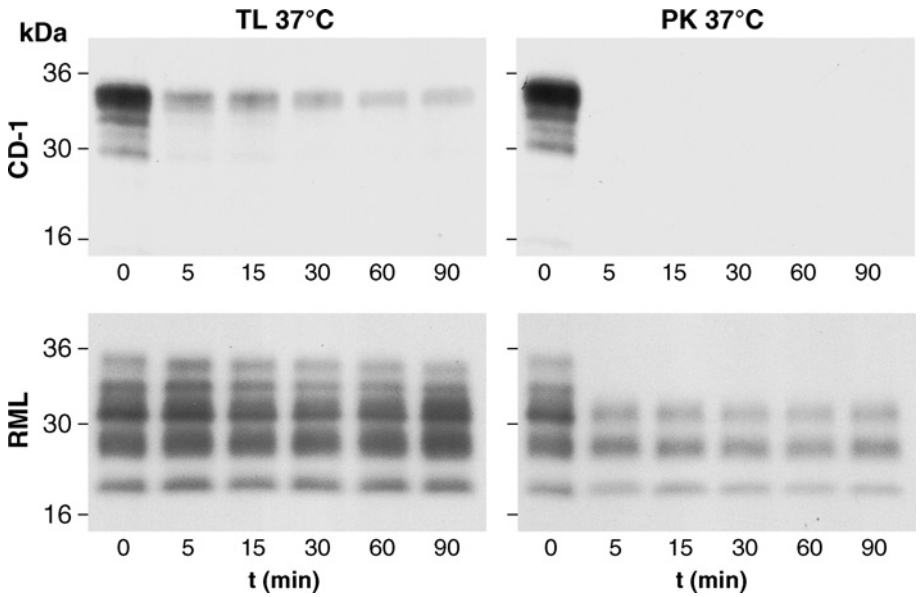

Figure 5 Immunoblot analysis of mouse PrP following digestion with thermolysin or PK at 37 °C

Normal CD-1 (CD-1) or RML (RML) brain homogenates [10 % (w/v)] were digested at 37 °C with thermolysin (TL 37 °C) (100 μg/ml) or PK (PK 37 °C) (50 μg/ml) for a range of incubation times (t). Equivalent aliquots were analysed by immunoblotting with the anti-PrP monoclonal antibody ICSM35. Molecular masses are stated on the left-hand side (in kDa).

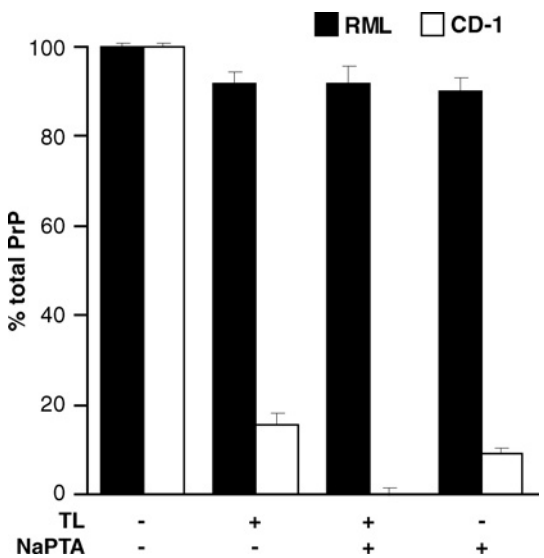

Figure 6 Thermolysin-resistant PrP from RML brain is precipitated by NaPTA

Homogenates from normal CD-1 brain (CD-1, white bars) or RML brain (RML, black bars) [10 % (w/v)] were either untreated (−) or treated (+) by digestion with thermolysin (TL) (100 μg/ml, 37 °C, 90 min), precipitated by NaPTA, or sequentially digested with thermolysin (100 μg/ml, 37 °C, 90 min) followed by NaPTA precipitation. NaPTA pellets were resuspended to their original volume in PBS. The concentration of PrP in all samples was measured by ELISA and is expressed as a percentage of the total PrP present in the respective untreated samples. Results are means ± S.E.M. ($n = 3$).

the starting infectivity was present in both samples. These results indicate that the majority of PK-sensitive thermolysin-resistant disease-related PrP isoforms do not contribute significantly to RML prion infectivity in cell culture. Immunoblot analysis (Figure 5) showed that while thermolysin produced no apparent change in the PrP fragment pattern over the digestion time course, PK rapidly produced the characteristic pattern of N-terminally truncated PrP fragments. The close similarity of prion infectivity seen in both thermolysin-digested and PK-digested samples suggests that removal of the N-terminus of PrP with PK does not adversely affect RML prion infectivity, at least as determined by the scrapie cell assay.

Thermolysin digestion of PrP associated with human and hamster prion strains

In a further series of experiments, we investigated the ability of thermolysin to degrade PrP^C while preserving disease-related isoforms of PrP associated with other prion strains. Under conditions that efficiently degrade hamster and human PrP^C (Figures 7a and 7b), preservation of intact PrP^{Sc} was observed in brain homogenate from patients with vCJD or terminally clinically affected hamsters propagating the Sc237 prion strain (Figures 7a and 7b). The presence of full-length PrP^{Sc} in these samples was confirmed by immunoblotting with the monoclonal antibody SAF32 (Figure 7c) or deglycosylation with PNGase F (results not shown). These findings, combined with the results of Owen et al. [23], establish that thermolysin can be used to isolate full-length PrP^{Sc} from multiple prion strain/host combinations.

While the presence of PK-sensitive disease-related PrP has been documented previously in many prion strain/host combinations, including prion-infected hamster brain [12,16,20], prion-infected mouse brain [38,39], scrapie-infected sheep brain [21] and patients with sporadic CJD [18], this has not been reported in vCJD brain. Accordingly, using ELISA, we quantified disease-related PrP in 10 % (w/v) brain homogenates from four patients with vCJD (Figure 8). Remarkably, up to 90 % of total PrP was resistant to digestion with thermolysin under conditions that efficiently degrade PrP^C in normal human brain (Figure 8). In contrast, only ~10–20 % of total PrP in vCJD brain resisted digestion with PK. This ratio was highly reproducible in all four patients, with mean percentages of thermolysin-resistant PrP and PK-resistant PrP (± S.D.) of 83 ± 13 % and 19 ± 6 % respectively.

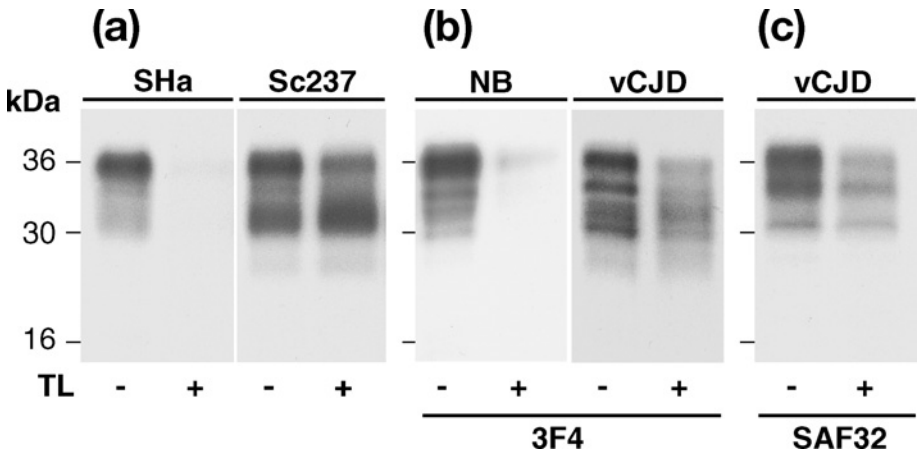

Figure 7 Thermolysin digestion of human and hamster brain homogenate

Homogenates from normal Syrian hamster brain (SHa), Sc237-infected Syrian hamster brain (Sc237), normal human brain (NB) or vCJD brain (vCJD) [10 % (w/v)] were analysed by immunoblotting before (−) or after (+) digestion with thermolysin (TL) (100 μg/ml, 70 °C, 1 h). Immunoblots were probed with the anti-PrP monoclonal antibodies 3F4 (**a**, **b**) or SAF32 (**c**) and were developed to show equivalent levels of PrP immunoreactivity in the TL (−) lanes. Molecular masses are stated on the left-hand side (in kDa).

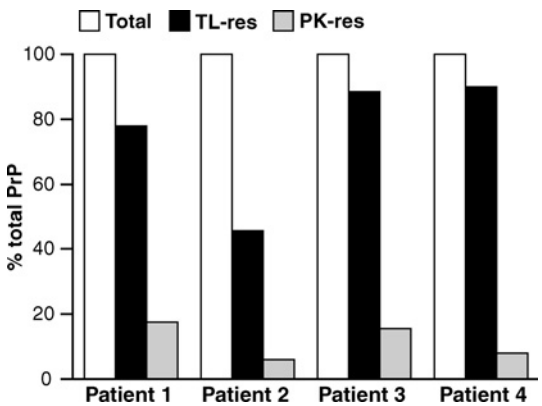

Figure 8 Predominance of thermolysin-resistant PrP in vCJD brain

PrP in 10 % (w/v) brain homogenates from four patients with vCJD was measured by ELISA in the absence of protease digestion (70 °C, 1 h) (Total, white bars) or after digestion with thermolysin (100 μg/ml, 1 h, 70 °C) (TL-res, black bars) or PK (100 μg/ml, 1 h, 37 °C) (PK-res, grey bars). PrP concentration is expressed as a percentage of the total PrP and the results are means ($n = 4$) with S.D. <10 %. The proportion of PK-sensitive PrP species present after thermolysin digestion was estimated by the ratio (TL-res − PK-res)/TL-res and was ∼80 % in samples from all four vCJD patients.

DISCUSSION

It is becoming increasingly clear that the pathogenesis of both human and animal prion diseases involves the propagation of protease-sensitive disease-related isoforms of PrP [12,16–21]. Indeed, in multiple prion strain/host combinations it now appears that the majority of disease-related PrP may be destroyed by PK under conditions that are typically employed to detect prototypical PrP^{Sc} [18,21]. PK-sensitive disease-related forms of PrP have been demonstrated by a conformation-dependent immunoassay [12,18,21,38,39] and by immunoblotting following biochemical purification [16,20], cold PK digestion [17] or immunological capture [19].

To date, little is known about the physicochemical properties of PK-sensitive disease-related PrP, and it remains unclear whether PK-sensitive and PK-resistant disease-related PrP species are conformationally distinct or simply comprise different-sized aggregates of essentially the same PrP conformers [16,20].

In the present study, we now report a simple method for detecting PK-sensitive disease-related PrP by showing that limited digestion with thermolysin not only preserves disease-related PrP in the full-length form [23], but also preserves a disease-related PrP fraction that is readily degraded by PK. In RML brain homogenate, under optimized conditions of proteolysis which destroy PrP^{C}, ∼85 % of total PrP resists degradation with thermolysin and is precipitated by NaPTA, whereas only ∼20 % of total PrP resists degradation with PK. Using the scrapie cell assay, which quantifies RML prion infectivity with a greater accuracy than a conventional mouse bioassay [33], we were able to quantify prion infectivity in these protease-digested samples. We found that the thermolysin-resistant PrP fraction, which preserves the majority of PK-sensitive disease-related PrP isoforms, contained the same level of infectivity as the PK-resistant PrP fraction, equivalent to ∼20 % of starting prion infectivity. Thus while the remaining RML prion infectivity appears to be roughly proportional to the concentration of PK-resistant PrP, there is a clear uncoupling of disease-related PrP concentration and prion infectivity in thermolysin-digested samples. We conclude from these results that the majority of PK-sensitive disease-related PrP isoforms are non-infectious, at least when measured by the scrapie cell assay.

The finding that both PK- and thermolysin-digested brain homogenates contain only ∼20 % of the starting prion infectivity despite thermolysin-digested samples containing ∼85 % of total homogenate PrP can be interpreted in several ways. Because the direct inactivation of thermolysin-resistant PrP by the protease seems unlikely, given the stability of the observed PrP fragment size, two other possibilities seem more plausible: (i) that ∼80 % of RML infectivity is associated with a minor population of PrP conformers that are equally sensitive to degradation by PK and by thermolysin, or (ii) that a non-PrP component of infectious prions that modifies specific prion infectivity is more efficiently degraded by thermolysin than by PK. Of these two possibilities, the former is more strongly supported by the findings of other researchers. Caughey and colleagues have demonstrated that hamster 263K prions have different-sized PrP aggregates possessing markedly different specific prion infectivities [40]. Small PrP aggregates comprising 14–28 PrP molecules, which represent only a minor proportion of total hamster PrP, have specific infectivities ∼70-fold higher than large PrP aggregates [40]. A similar physical

distribution of infectious PrP aggregates associated with the RML prion strain would provide a reasonable physical basis for our findings. Given that small PrP aggregates appear to be more readily accessible to proteolytic degradation [16,20], both thermolysin and PK may equivalently destroy a subset of PrP species corresponding to the most infectious prions that account for up to 80 % of total infectivity.

The recent results of Requena and colleagues have established that the PK-sensitive disease-related hamster PrP isolated from the upper fractions of sucrose-density gradients has the ability to convert PrP^C into protease-resistant PrP using the PMCA (protein misfolding cyclic amplification) assay [20]. Importantly, because PK-sensitive disease-related hamster PrP was isolated on the basis of density [20], these preparations are unlikely to contain the full ensemble of PK-sensitive PrP species. While there are of course caveats when comparing different prion strains and different methods, our results suggest that the PK-sensitive PrP species are heterogeneous and that the majority may be non-infectious.

There is increasing evidence that an abnormal PrP species, distinct from PrP^{Sc}, may be responsible for neurotoxicity in prion disease [7]. Clearly, purification of thermolysin-resistant PK-sensitive disease-related PrP is now required for detailed evaluation of its specific infectivity in a mouse bioassay and its possible contributions to neurotoxicity or other roles in prion disease pathogenesis.

Safar et al. [18] reported previously that 80–90 % of disease-related PrP in sporadic CJD brain is destroyed by PK and that detection of this fraction by conformation-dependent immunoassay enhances diagnostic sensitivity. Our finding that up to 90 % of total PrP in vCJD brain homogenate resists digestion with thermolysin under conditions that destroy human PrP^C verifies the proposal [23] that thermolysin may be useful in the development of high-sensitivity diagnostic tests. Thermolysin may also be useful in identifying unusual PrP^{Sc} isoforms in isolates where undetectable or barely detectable levels of PK-resistant PrP are observed; for example, in certain inherited prion diseases [2,41,42] and in atypical prion disease of ruminants [43,44]. More recently, Owen et al. [24] have reported the potential value of thermolysin for prion strain discrimination based upon the variable generation of truncated PrP fragments. Our results obtained with two distinct prion strains (mouse RML and human vCJD prions) suggest that measuring the ratio of PK-resistant disease-related PrP to thermolysin-resistant disease-related PrP may also be informative in this regard.

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