Structural Organization of Mammalian Prions as Probed by Limited Proteolysis

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Abstract

Elucidation of the structure of PrPSc continues to be one major challenge in prion research. The mechanism of propagation of these infectious agents will not be understood until their structure is solved. Given that high resolution techniques such as NMR or X-ray crystallography cannot be used, a number of lower resolution analytical approaches have been attempted. Thus, limited proteolysis has been successfully used to pinpoint flexible regions within prion multimers (PrPSc). However, the presence of covalently attached sugar antennae and glycosylphosphatidylinositol (GPI) moieties makes mass spectrometry-based analysis impractical. In order to surmount these difficulties we analyzed PrPSc from transgenic mice expressing prion protein (PrP) lacking the GPI membrane anchor. Such animals produce prions that are devoid of the GPI anchor and sugar antennae, and, thereby, permit the detection and location of flexible, proteinase K (PK) susceptible regions by Western blot and mass spectrometry-based analysis. GPI-less PrPSc samples were digested with PK. PK-resistant peptides were identified, and found to correspond to molecules cleaved at positions 81, 85, 89, 116, 133, 134, 141, 152, 153, 162, 169 and 179. The first 10 peptides (to position 153), match very well with PK cleavage sites we previously identified in wild type PrPSc. These results reinforce the hypothesis that the structure of PrPSc consists of a series of highly PK-resistant β-sheet strands connected by short flexible PK-sensitive loops and turns. A sizeable C-terminal stretch of PrPSc is highly resistant to PK and therefore perhaps also contains β-sheet secondary structure.

Introduction

Prions are the etiological agents responsible for a diverse set of transmissible fatal neurodegenerative diseases of humans and animals, characterized by an abnormal accumulation of prion protein (PrP) [1,2], primarily in the brain. Prions replicate by converting the normal non-infectious cellular prion protein (PrPC) into a prion (PrPSc), via a poorly characterized post-translational conformational transformation. In mice, PrP contains approximately 209 amino acids (numbered 23–231 after cleavage of a 22–mer signal peptide) and has four covalent post-translational modifications: two asparagine N-linked glycans at residues N 180 and N 196, a disulphide bridge between residues C 178–C 213 and a glycosylphosphatidylinositol (GPI) anchor attached to the C-terminus of the protein (residue S 231) [2,3]. Mouse PrPC is a monomer, while PrPSc is a heterogeneous multimer [2,3]. There have been no demonstrated conformational differences between mouse PrPSc and PrPSc. The only difference between PrPSc and PrPC is conformational; they are isoforms [2].

The structure of folded, monomeric recombinant PrP, highly likely to be identical to that of PrPSc, has been solved by NMR spectroscopy [4] and X-ray crystallography [5]. In contrast, the structure of PrPSc remains unclear because the insolubility of PrPSc and the failure to crystallize the heterogeneous PrPSc multimers prevent the application of the mentioned high resolution analytical techniques. However, a variety of lower resolution instrumental techniques have provided some information about the structure of PrPSc. Unlike PrPSc, PrPSc is partially resistant to proteinase K (PK) digestion [2,6]. The secondary structure of PrPSc is largely composed of unstructured and α-helical regions, while PrPSc is largely composed of β-sheet with little, if any, α-helix [7,8,9]. The structure of PrPSc has also been studied using electron microscopy-based analysis of two-dimensional crystals of the PK resistant core of Syrian hamster (SHa) PrPSc (PrP27–30) [10,11] and mass spectrometry (MS)-based analysis of hydrogen/deuterium exchange [9]. Although theoretical models for PrPSc have been proposed [10,12], there is an insufficient amount of experimental data to reach a definitive consensus.

In a previous study, we used limited proteolysis to elucidate structural features of PrPSc [13]. Conformational parameters such as surface exposure of amino acids, flexibility, and local interactions correlate well with limited proteolysis. Peptide bonds located within β-strands are resistant to proteolytic cleavage, whereas peptide bonds within loops and, more rarely, α-helices may be cleaved [14]. Therefore, the targets for limited proteolysis

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are locally unfolded or highly flexible segments [14]. In our previous study [13], we demonstrated the usefulness of combining limited proteolysis and mass spectrometry (MS) to obtain structural information about two strains of hamster PrP Sc. We concluded that the amino-terminal half of PrP Sc features a series of short PK-resistant stretches, presumably β-strands, interspersed with short PK-sensitive stretches, likely loops and turns. Unfortunately, the structural information was largely limited to the N-terminal portion of the protein, as a consequence of the covalent attachment of the heterogeneous GPI anchor and the heterogeneous asparagine-linked sugar antennae to amino acids in the C-terminal portion of the molecule, which prevented MS-based analysis of this part of the molecule.

Here we extended our studies of the structure of PrP Sc, by using transgenic (tg) mice expressing PrP Sc lacking the GPI anchor (GPI−) [15]. The GPI−PrP Sc produced by these mice is fully infectious, lacks the GPI anchor, and is largely unglycosylated, which reduces the heterogeneity in the C-terminal portion of the molecule [15,16]. These properties make it ideal to carry out structural studies, and have allowed us to obtain, for the first time, a complete survey of the whole PrP Sc sequence, regarding its susceptibility to proteolysis.

Results

Accumulation of PrP Sc in GPI-anchorless Mice

Homozygous GPI-anchorless PrP mice were inoculated at 6 weeks of age with the RML strain of murine-adapted scrapie. Three-hundred sixty-five days post-inoculation, the mice were humanely euthanized. Their brains were surgically removed for further biochemical processing. The presence of PrP Sc was confirmed by digesting a portion of some of these brains, after suitable homogenization, with proteasome K (PK) and analyzing the result by Western blot (Figure 1A and S1). The PK treatment yielded the characteristic PK resistant core protein, referred to as PrP27-30 in PK-treated wild-type PrP Sc, although in this case its yield was limited proteolysis and mass spectrometry (MS) to obtain structural information about two strains of hamster PrP Sc. We concluded that the amino-terminal half of PrP Sc features a series of short PK-resistant stretches, presumably β-strands, interspersed with short PK-sensitive stretches, likely loops and turns. Unfortunately, the structural information was largely limited to the N-terminal portion of the protein, as a consequence of the covalent attachment of the heterogeneous GPI anchor and the heterogeneous asparagine-linked sugar antennae to amino acids in the C-terminal portion of the molecule, which prevented MS-based analysis of this part of the molecule.

We isolated PK-resistant PrP Sc fragments from infected GPI− mice. Purity of this material was assessed by SDS-PAGE followed by Coomassie staining (Figure S2). Using a high resolution Tricine/SDS-PAGE system [17], we compared the distribution of these fragments with that of fragments present in PK-treated unpurified GPI− infected brain homogenate, and found them to be similar, which demonstrates that our purification process isolates all of the PK-resistant fragments (Figure S3). GPI− PrP Sc, unlike wild-type PrP Sc, permits the use of MS to accurately identify all PK cleavage sites. This allowed us to analyze samples by Western blot (WB) and by MS.

We analyzed our samples with high mass accuracy using nano-LC-ESI-QqQ-TOF MS (Figure S4) and identified three peaks of 17149, 16728, and 16371 Da (peptides G81-S232, G85-S232, and G92-S232). The smaller peptides were analyzed by MALDI-TOF. MS-based analysis revealed that the seven bands present in the WB (vide infra) contained thirteen peptides with MWs of 17140, 16726, 16371, 13066, 13463, 12173, 12041, 11171, 9687, 9573, 8359, 7436 and 6274 Da. By comparing the observed masses with those calculated from the mouse GPI PrP sequence, we determined that they correspond to peptides G81-S232, G85-S232, G92-S232, A106-S232, G118-S232, M135-S232, S134-S232, G141-S232, N152-S232, M153-S232, Y162-S232, S169-S232, and V173-S232 (Figure 2 and Table 1). No C-terminally truncated peptides were observed in our MS or WB-based analysis (vide infra).

Identification of PK Cleavage Sites in GPI-anchorless PrP Sc by Western Blot

In parallel we used Tricine-SDS-PAGE [17] followed by WB to analyze the PK-digested GPI− PrP Sc (Figure 3). When the WB was probed with the antibody #51 (epitope G92-K100), just one wide band (~17 kDa) was observed, suggesting a set of cleavage products near G92 with no C-terminally truncated fragments. A blot probed with the W226 antibody (epitope W144-N152), revealed three additional faint bands (~14.6, 13 and 12 kDa), suggesting three PK cleavage sites between the epitopes of these antibodies. Probing with the C-terminal R1 antibody (epitope Y227-S230) revealed three more bands (~10.2, 8 and 6.7 kDa), suggesting three additional cleavage sites near residues Y148, F164 and V173.

These bands agree quite well with our MS-based analysis (vide supra). In order to exclude the possibility that the observed PK-resistant fragments are the result of the known preference of PK of certain amino acid residues, rather than structural constraints, we subjected a similar amount of freshly refolded, recombinant MoPrP to cleavage by PK. A concentration of PK much lower than that used with mouse GPI− PrP Sc, 1 μg/ml, completely destroyed all PrP, leaving no PK-resistant fragments larger than 3.5 kDa (Figure S5). Only PK concentrations below 1 μg/ml yielded some partially resistant fragments, whose sizes do not match those of PK-treated GPI− PrP Sc.

Kinetics of PK Digestion in GPI-anchorless PrP Sc

We performed a PK-digestion time course to determine the relationship of these peptides to one another. A time-dependent reduction in intensity of all PK-resistant bands was observed (Figure 4). The intensities of the 17, 14.6, 13, 12, and 6.7 kDa bands decreased steadily over time. By 240 minutes the intensities of the 17 and 10.2 kDa bands are nearly equal and by 360 minutes the intensity of the 17, 10.2 and 8 kDa bands are similar. These results are consistent with a progressive digestion of GPI− PrP Sc from the N-terminus. This further suggests that different PK-resistant fragments are not from different sub-populations of GPI− PrP Sc, instead they are derived from a larger common GPI− PrP Sc peptide.

PK Cleavage Analysis After Partial Unfolding of GPI-anchorless PrP Sc

The above observations were confirmed when the GPI− PrP Sc was partially unfolded with increasing concentrations of guanidinium prior to PK cleavage, following the procedure of Kocisko et al. [18]. These authors have shown that partial unfolding of PrP Sc with up to 2.5–3 M guanidinium is reversible upon dialysis. GPI−
PrPSc became more susceptible to proteolytic digestion in a guanidine-concentration dependent manner. At concentrations above 1 M, the 10.2 and, to a lesser extent, 12 and 8 kDa bands (N152-S232/M153-S232, G141-S232, and Y162-S232) predominate. Above 3 M guanidine, which renders the unfolding irreversible [18], almost no PK-resistant material remains (Figure 5). These results mirror those of the PK time course (vide supra), i.e. all of the bands are derived from the progressive N-terminal digestion of a progenitor peptide. In their original report, Kocisko et al. identified in SHaPrPSc partially unfolded with guanidine, a highly stable PK-resistant core starts before position 143 and continues to the C-terminus [18]. Sajnani et al. also detected a resistant SHaPrPSc core starting at position 139/142 [13].

Discussion

We present a complete survey of susceptibility to limited proteolysis of a PrPSc strain (Figure S6). The map of PK-susceptible spots: 116–118, 133–134, 141, 152–153, 162, 169, and 179, strongly suggests regions corresponding to loops and turns, while nicks at 81, 85, and 89 signal the frontier between the structured C-terminal and unstructured N-terminal domains of PrPSc. Given the high proportion of β-sheet secondary structure derived from FTIR analyses, it is logical to conclude that PK-resistant stretches flanking these spots most likely are strands of β-sheet.

Our results are in excellent agreement with our previous studies of wild-type PrPSc [13]. Our experiments with two different SHaPrPSc strains showed the sequence stretches 23–86 (263K), 23–101 (Dy), 117–119, 131–142, and the region around 154 (= mouse M153) to be sensitive to PK. In the present study, besides confirming these regions as being PK-sensitive, we identified three additional PK cleavage sites in the C-terminal region of GPI-PrPSc (Y162, S169 and V179).

We did not find evidence of any PK-resistant peptide with an N-terminus beginning beyond V179. This is not a consequence of technical limitations, since the Tricine-based SDS-PAGE allows identification of peptides as small as 3.5 kDa (Figure 3). Instead, either this region is completely resistant to PK, or no stable PK-resistant cores remain if PK cleaves beyond that point.

Our results also agree with several studies describing amino-terminally truncated PK-resistant peptides in human CJD PrPSc.

Figure 1. Characterization of GPI-PrPSc. A. Western blot of brain homogenate from scrapie-infected GPI- tg mouse before and after digestion with PK (25 μg/ml); WB probed with SAF83 antibody. B. Histopathological and immunohistochemical analyses of scrapie-infected GPI- tg mouse brain. (a) Haematoxylin-eosin staining of the hippocampal formation. (b) IHC staining (antibody 6H4) of the hippocampal formation. C. Kaplan-Meier survival curves of wild-type mice (C57BL/6) inoculated with 2% of brain homogenate from scrapie-infected GPI-PrPSc (green line) and a negative control inoculated with PBS (red line). doi:10.1371/journal.pone.0050111.g001
Figure 2. MALDI-TOF spectrum of PK-treated purified GPI\(^{2}\) PrP\(^{Sc}\). Doubly-charged ions from peptides with m/z 16371 and 17148 are indicated (*). Low resolution in the >16 kDa region precluded identifying unmarked peaks. A scheme of GPI\(^{+}\) PrP sequence with PK cleavage points (color coded) and secondary structure of PrP\(^{Sc}\) is included at the top: (octarepeats (□), β-sheets (●), and α-helices (□)); epitopes of the mAbs used are also indicated.

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Table 1. PK-resistant fragments in GPI\(^{−}\) PrP\(^{Sc}\).

| WESTERN BLOT | MALDI-TOF |
|--------------|-----------|
| **Band**     | **kDa**   | **Peak (Da)** | **Theoretical mass (Da)** | **Cleavage point** | **Peptide** |
| 1            | 17        | 17148 16726 16371 | 17148 16729 16371 | 81 85 89 | G\(_{81}\) - S\(_{85}\) G\(_{85}\) - S\(_{89}\) |
| 2            | 14.6      | 13606 13463 | 13605 13463 | 116 118 | A\(_{116}\) - S\(_{118}\) |
| 3            | 13        | 12173 12041 | 12172 12041 | 133 134 | M\(_{133}\) - S\(_{134}\) |
| 4            | 12        | 11171 | 11172 | 141 | G\(_{143}\) - S\(_{232}\) |
| 5            | 10.2      | 9687 9573 | 9688 9574 | 152 153 | N\(_{152}\) - S\(_{153}\) M\(_{153}\) - S\(_{153}\) |
| 6            | 8         | 8358 | 8358 | 162 | Y\(_{162}\) - S\(_{232}\) |
| 7            | 6.7       | 7436 6274 | 7436 6278 | 169 179 | S\(_{169}\) - S\(_{232}\) V\(_{179}\) - S\(_{232}\) |

*Entries sharing a color represent PK-resistant peptides of very similar MW that were not resolved on the tricine gel.

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PrPSc share key structural elements, which would explain the being resistant to PK [21]. This suggests that synthetic prions and 138/141, 152/153, and 162, and extending to the C-terminus as antibody.

60, 120, 180, 240, 300 and 360 minutes. Samples were treated with PNGase F and subjected to Tricine-SDS-PAGE the blot was probed with R1

et al mouse prions, Bocharova et al. described two additional amino-terminally truncated human CJD PrPSc peptides (MW of 16/17 kDa) [20], respectively. Zanusso et al. described two additional amino-terminally truncated human CJD PrPSc peptides (MW of 16/17 kDa) [20], analogous to the GPl PrPSc peptides G141-S232 and M133-S232, respectively. Zanussi et al. described two additional amino-terminally truncated human CJD PrPSc peptides (MW of 16/17 kDa) [20], analogous to the GPl PrPSc peptides G141-S232 and M133-S232, respectively. Kocisko et al. used a C-terminal antibody (epitope 217-232) to demonstrate the presence of a number of amino-terminally truncated PK-resistant species in SHaPrPSc [18]. Using synthetic mouse prions, Bocharova et al. identified the regions beginning at 138/141, 152/153, and 162, and extending to the C-terminus as being resistant to PK [21]. This suggests that synthetic prions and PrPSc share key structural elements, which would explain the capacity of recombinant PrP fibrils to change their conformation, via a “deformed templating” mechanism, to that of PrPSc [22].

In contrast, relatively few C-terminally truncated peptides have been described. Notari et al. reported two human CJD PrPSc peptides truncated near position 228 [23]. Stahl et al. also reported the presence of a peptide truncated at position 228 in PK-treated SHaPrPSc [24]. The absence of such fragments in our study could be explained by slight differences in sample preparation, or perhaps by the fact that the absence of the GPI-anchor might have an effect on nearby residues.

This conspicuous absence of the C-terminally truncated peptides is a reflection of the stability of the C-terminal region, in GPl PrPSc appears to be the most stable part of the molecule, which is inconsistent with the presence of substantial stretches of α-helical secondary structure in that region. Our results agree with Smarrnov et al., who showed the C-terminus of GPl PrPSc to exhibit extremely low rates of H/D exchange, typical of extensive H-bonding (β-sheet) [9]. These authors showed that an FTIR absorbance band (∼1,660 cm⁻¹) previously assigned to α-helical secondary structure in PrPSc is also present in the spectrum of recombinant PrP amyloid fibrils, which contain no α-helices, and therefore cannot be taken as evidence of the presence of α-helical structure. They concluded that GPl-PrPSc consists of a series of β-sheet stretches connected by short loops and/α turns, in agreement with our conclusions. Some stretches exhibiting a somewhat higher exchange rate, suggested to overlap with loops/turns, such as 133-148 or 81-118, are consistent with flexible stretches identified in our study, although discrepancies also exist. The limited resolution of both analytical techniques prevents a more exhaustive comparison, but overall both of them agree.

GPl PrPSc fibrils are about 3–5 nm wide [25] and our unpublished results). This constraint means that each PrPSc monomer must be coiled in such a way as to fit approximately 140–145 residues (∼G85–S232) into this width. To do so, PrPSc monomers must necessarily adopt a multi-layer architecture, as seen in SH3 fibers [26] or the HET-s fungal prion domain [27]. The HET-s prion domain packs 70 residues into two β-strands alternating with turns and loops [27]. Wille et al. have suggested that PrPSc fibrils are composed of four rungs of β-strands, based on their interpretation of X-ray diffraction patterns [28]. In this model, each rung would comprise ∼36–37 residues. Positions N152-M153 lie near the middle of the G85–S232 sequence, so it is tempting to speculate that they might be located at an exposed position at the border between rungs. This might explain why the N152-S232 and/or M153-S232 fragment emerges as the most conspicuous PK-resistant fragment after prolonged treatment with PK or partial unfolding with guanidine (Figures 4 and 5). Positions A116-G118 might be the border between the two most amino-terminal rungs (approximately G85-A115 and A115-E151). On the other hand, our results are partially inconsistent with the location

Figure 3. Western blot analysis of PK-resistant GPl PrPSc. Unpurified GPl PrPSc was treated with 25 μg/ml of PK and subsequently deglycosylated with PNGase F. Samples were resolved on Tricine-SDS-PAGE and probed with the monoclonal antibodies, #51 (lane 1), W226 (lane 2), and R1 (lane 3).
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Figure 4. Kinetics of PK digestion of unpurified GPl PrPSc. Samples were digested with PK (25 μg/ml) and the reaction stopped after 0, 30, 60, 120, 180, 240, 300 and 360 minutes. Samples were treated with PNGase F and subjected to Tricine-SDS-PAGE the blot was probed with R1 antibody.
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expression of GPI- PrP confirmed by Western blot (Figure S1). By Chesebro
to obtain homozygous GPI- animals (tg44
Mountain Laboratories, NIH, Montana, USA. Mice were crossed (tg44(
K100-P104 and E145-R163, placed in loops and not rungs [10]. Our
rung (Figure 2 and Table 1).
composited primarily of
b
strains) and those of other researchers using SHaPrPSc. Further-
our data are consistent with our previous results (263K and Dy
PrPSc, which suggests that the myriad human, hamster and mouse
more, they are consistent with those observed for human CJD
refolded protein was dialyzed against 10 mM sodium phosphate buffer pH 5.8 and then against d.i. water.

Limited Proteolysis
aliquote of BH (10% in PBS, 5% Sarkosyl) were digested with
PK (Sigma-Aldrich, St. Louis, MO, USA) in 20 nM Tris-HCl pH 8.5 at 37°C for 1 h unless otherwise stated. Digestion was
stopped by addition of Pefabloc (Fluka, Buchs, Switzerland) to a final concentration of 2 mM. Deglycosylation was carried out with
2 μl of PNGase F solution (New England Biolabs, Ipswich, MA, USA) at 37°C for 48 h, according to the manufacturer’s instructions.

Digestion with PK After Partial Unfolding with Guanidine HCl (Gnd)
Samples of BH (5 μl) were mixed with an equal volume of an appropriate aqueous Gnd solution to yield the desired final Gnd concentration and then incubated at 37°C for 1 h. After incubating, the samples were diluted with buffer (20 mM Tris-HCl pH 8.5) to yield a 0.4 M Gnd solution, which were then treated with PK (25 μg/ml) for 1 h at 37°C. The digestion was stopped by adding Pefabloc (2 mM final concentration) and the protein was precipitated by addition of ice-cold methanol (85% final concentration). The resulting pellets were resuspended in 9 μl of deionized water, and deglycosylated with PNGase F (vide supra).

Tricine-SDS-PAGE and Western Blot Analysis
The precipitated pellets were boiled for 10 minutes in 10 μl of Tricine sample buffer (BioRad, Hercules, CA, USA) containing
2% (v/v) of β-mercaptoethanol. Electrophoresis was performed using precast 10-20% Tris-Tricine/Peptide gels (BioRad, Hercule-
es, CA, USA), in the Criterion System (BioRad, Hercules, CA, USA). The cathode buffer was Tris-Tricine-SDS buffer 1 ×
(Sigma-Aldrich, St. Louis, MO, USA) and the anode buffer, 1 M Tris-HCl pH 8.9. Electrophoresis was performed at constant voltage (125 volts) for 200 minutes, on ice.

The gels were electroblotted (350 mA, for 150 minutes; 4°C) onto PVDF membranes (Immobilon-P, 0.45 μm; Millipore, Bill-
erica, MA, USA). Membranes were probed with the following monoclonal antibodies: mAb #51 (epitope: G92-K100), undiluted; W226 (epitope: W144-N152), at 1:5000 dilution; or R1 (epitope: Y225-S230), at a 1:5000 dilution. Peroxidase-conjugated anti-mouse or anti-human antibodies (GE Healthcare, Little Chalfont, UK)
were used as a secondary antibody, as appropriate (1:5000 dilution). Blots were developed with ECL-plus reagent (GE Healthcare, Little Chalfont, UK). Three sets of partially overlapping MW markers, Peptide Molecular Weight (Sigma-Aldrich, St. Louis, MO, USA), Kaleidoscope Prestained Standard (BioRad, Hercules, CA, USA) and Novex Sharp Protein Standard (Invitrogen, Carlsbad, CA, USA) were run in each analysis to calibrate the MW of the bands.

Mass Spectrometry
NanoLC/ESI/MS analysis was done with an Applied Biosystems (AB SCIEX, Framingham, MA) model QStar Pulsar equipped with a Proxeon Biosystems (Odense, Denmark) nanoelectrospray source. Samples of the Gnd stock solution (vide supra) were loaded automatically onto a C-18 trapping cartridge and chromatographed on a reversed-phase column (Vydac Everest 2383V5,02515, 75 mm × 150 mm) fitted with a coated spray tip (FS360-50-5-CE; New Objectvie, Inc.). A nanoflow LC system (Dionex, Sunnyvale, CA) with autosampler, column switching device, loading pump, and nanoflow solvent delivery system was used. Elution solvents were A (0.5% acetic acid in water) and B (0.5% acetic acid in 80% acetonitrile/20% water). Samples were eluted at 250 nL/min using a binary gradient (8% B at 0 min to 80% B in a 30 min linear gradient, held at 80% B for 5 min, then back to 8% B for 15 minutes). The QStar Pulsar was externally calibrated daily with human [Glu1]-fibrinopeptide B.

In parallel, 1 μL of the Gnd stock solution was mixed with 49 μL of sinapinic acid (SA) solution (10 mg/mL, SA dissolved in 30% ACN with 0.3% TFA) and analyzed by MALDI-TOF. One half μL aliquots were deposited using the dried-droplet method onto a 384 Opti-TOF MALDI plate (Applied Bio- systems, Foster City, CA, USA). MALDI analysis was performed in a 4800 MALDI-TOF/TOF analyzer (Applied Biosystems, Foster City, CA, USA). MS spectra were acquired in linear mode (20 kV source) with a NdYAG (355 nm) laser, and averaging 500 laser shots. The mass of the peptide M_{1135-232} (9573 Da) was determined by an iterative calibration approach, using insulin (m/z = 5733), ribonuclease A (m/z = 13682) and lysozyme (m/z = 14305), (Sigma-Aldrich, St. Louis, MO) as internal standards. Then, the signals from the M_{1135-232} (9573 Da), G_{90-232} (16371 Da), and G_{111-232} (17148 Da) peptides were used to calibrate the rest of peaks in the spectrum. Masses were matched to PrP fragments with the help of GPMAW 6.0 software (Lighthouse, Odense, Denmark).

Immunohistochemistry
Immediately after extraction, the brain was fixed in formalin and then sliced into four transversal sections by cutting the brain caudally and rostrally to the midbrain and at the level of the basal nuclei. The sections were dehydrated by equilibration in solutions of progressively higher ethanol concentration and then equilibrated with xylene before being embedded in paraffin. Haematoxylin-eosin was used to stain the 4 μm thick sections. Additional sections were mounted on 3-triethoxysilyl-propylamine-coated glass slides for immunohistochemical (IHC) studies.

These brain sections were deparaffinised, immersed in formic acid containing peroxidase inhibitors, and autoclaved prior to IHC analysis. These autoclaved samples were washed, treated with proteinase K, washed again, and then incubated overnight with the antibody 6H4 (1:2000, Prionics AG, Schlieren, Switzerland). The sections were developed using the DAKO EnVision system and 3,3’-diaminobenzidine as the chromogenic substrate.

Supporting Information

Figure S1 Western blot of unpurified GPI-PrP_{Sc} --/+PK. Both samples were treated with PNGase F. WB was probed with the #51 antibody. (TIF)

Figure S2 Characterization of isolated GPI-PrP_{Sc}. 10 μl of sample were loaded and separated in a 15% gel by SDS-PAGE. The gel was stained by Coomassie blue. The molecular weight of the GIPI-less PrP_{27-30} is ~16750 Da. (TIF)

Figure S3 Western blot of PK-resistant fragments. In unpurified (1) and purified GPI-PrP_{Sc} (2). Both samples were digested with proteinase K, 25 μg/ml and 10 μg/ml respectively, treated with PNGase F and resolved on a Tricine-SDS-PAGE gel. WB was probed with the R1 antibody. (TIF)

Figure S4 Bayesian protein reconstruction of the nano-LC-ESI-MS spectra of PK-treated purified GPI-PrP_{Sc}. The mass graphs of the three peaks: 17148 Da (top), 16729 Da (middle) and 16371 Da (bottom), identified by ESI-TOF are shown. (TIF)

Figure S5 Western blot of recombinant MoPrP(23–231) cleavage by PK. Samples were digested with different concentrations of PK: 0, 0.2, 1, 5, 10 and 25 μg/ml. Samples were subjected to Tricine-SDS-PAGE and the blot was probed with R1 antibody. (TIF)

Figure S6 Schematic representations of the data. A. A scheme of GIP-PrP sequence, showing the PK-resistant areas (blue squares) and the PK cleavage points and flexible areas (gray line). B. Lengthwise comparison of the different peptides found by limited proteolysis and MALDI-TOF analysis (colors match those displayed in Figure 2). (TIF)

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Author Contributions
Conceived and designed the experiments: EVF JA CJS JRR. Performed the experiments: EVF JA EV ID. Analyzed the data: EVF JA EV CJS JRR. Performed the experiments: EVF JA EV ID. Analyzed the data: EVF JA EV CJS JRR. Contributed reagents/materials/analysis tools: MAP AR LS BP. Wrote the paper: EVF CJS JRR.

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