Small Protease Sensitive Oligomers of PrPSc in Distinct Human Prions Determine Conversion Rate of PrPC

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Abstract

The mammalian prions replicate by converting cellular prion protein (PrPC) into pathogenic conformational isoform (PrPSc). Variations in prions, which cause different disease phenotypes, are referred to as strains. The mechanism of high-fidelity replication of prion strains in the absence of nucleic acid remains unsolved. We investigated the impact of different conformational characteristics of PrPSc on conversion of PrPC in vitro using PrPSc seeds from the most frequent human prion disease worldwide, the Creutzfeldt-Jakob disease (sCJD). The conversion potency of a broad spectrum of distinct sCJD prions was governed by the level, conformation, and stability of small oligomers of the protease-sensitive (s) PrPSc. The tight correlation between conversion potency of small oligomers of human sPrPSc observed in vitro and duration of the disease suggests that sPrPSc conformers are an important determinant of prion strain characteristics that control the progression rate of the disease.

Introduction

The yeast, fungal, and mammalian prions determine heritable and infectious traits, and thus behave like proteinaceous genes [1]. In mammals, prions cause a group of fatal and rapidly progressive neurodegenerative diseases, originally described as transmissible spongiform encephalopathies (TSEs) [1,2]. The most common of the human prion diseases is sporadic Creutzfeldt-Jakob disease (sCJD) [3], accounting for ~85% of all CJD cases worldwide [1]. Although the recent progress in the understanding of yeast and rodent-adapted prions was remarkable, whether or to what extent these findings can be applied to human prions is unclear [4] and the origin and pathogenesis of sCJD remains enigmatic [1]. Moreover, the number of prion strains that cause sCJD is not known [4–6] and, in contrast to growing structural characterization of rodent prions [7–9], no direct structural data are available for pathogenic prion protein (PrPSc) present in sCJD brains beyond the evidence that it is variably resistant to proteolytic digestion [5,10,11].

Mammalian prion diseases were originally characterized by deposits of protease-resistant prion protein (PrPSc), often forming large amyloid plaques and fibrils [12,13]. Having a basic amino acid composition and an unstructured N-terminus, PrP can assume at least two conformations: native, α-helix-rich PrP0 [14] which is host-encoded by the chromosomal gene PRNP and expressed at different levels in mammalian cells [15]; and disease-causing, β-sheet-rich PrPSc [16,17]. The prion hypothesis based on these findings posited that mammalian prions replicate by converting host’s cellular prion protein (PrPC) into pathogenic protease resistant (r) conformational isofrom (PrPSc) [18]. However, the variable specific infectivity of rPrPSc and apparent absence of protease-resistant PrPSc or amyloid fibrils in growing number of prion diseases [11,19] lead some researchers to question the causative link between rPrPSc and prion infectivity [6,20,21]. Apart from generating a controversy, these findings have raised fundamental questions; specifically, whether the amyloid or amyloid fibrils cause the disease; whether protease-sensitive (s) forms of PrPSc [22] comprise the initial steps in prion replication or are related to the alternative misfolding pathway generating noninfectious aggregates [4,5]. Interestingly, subsequent experiments with purified and detergent-dissociated Syrian hamster PrPSc demonstrated a high seeding (replication) potency of small oligomers of the pathogenic prion protein [23]. Cumulatively, these findings raised an intriguing possibility that sPrPSc found invariably in sCJD-infected brains might be composed of such highly potent small oligomers.

Early important studies with mouse and Syrian hamster PrPC demonstrated that the infectious PrPSc can be amplified indefinitely in crude brain homogenates by using alternating rounds of sonication and incubation, a procedure called serial protein misfolding cycled amplification (sPMCA) [24]. Whether rPrPSc generated in PMCA is as infectious as the original brain derived sample is currently debated [25] but subsequent experiments with
Author Summary

Mammalian prion diseases were originally characterized by accumulation of protease-resistant prion protein (PrPSc), often forming large amyloid deposits and fibrils. However, the apparent absence of protease-resistant PrPSc or amyloid fibrils in growing number of prion diseases raised several fundamental questions: specifically, whether presumably protease-sensitive forms of PrPSc exist as distinct conformers; and whether they comprise the initial steps in prion replication or are related to the alternative misfolding pathway generating noninfectious aggregates. We investigated the conformational characteristics of protease sensitive conformers of PrPSc and their role in the pathogenesis of sporadic Creutzfeldt-Jakob disease (sCJD). Using two different in vitro prion protein (PrPPr) conversion techniques in tandem with biophysical methods, we identified small oligomers of protease sensitive PrPSc present in sCJD brains as the most potent initiators of PrPSc conversion. Their concentration and conformational stability determine the distinctly different replication potency of PrPSc in individual isolates of sCJD and each of these characteristics correlates tightly with duration of the disease. These features argue for a broad range of distinct prion strains causing the sCJD and imply that small oligomers of protease sensitive conformers of pathogenic prion protein are encoding incubation time and progression rate of the disease.

rodent, ungulate, and human prions proved that the procedure faithfully replicates the qualitative characteristics of various prion isolates [26]. In a parallel development, purified bacterially expressed recombinant (rec) PrP was shown to be converted by infectious rPrPSc in sonication-driven or quaking-induced conversion (QuIC), and yielded protease-resistant aggregates with a PK digestion pattern closely related to original brain PrPSc [27,28]. Despite the low infectivity of recombinant replicas of Syrian hamster PrPSc, these approaches helped to define some key elements of prion structure [27–29] and have shown specific and quantitative response to the brain-derived PrPSc used as a seed [30–32]. Although the PMCA and analogous techniques allowed to create prions “de novo” from recombinant proteins and thus prove in principle that mammalian prions are misfolded proteins [33–35], the remarkably precise mechanism replicating conformational features of PrPSc and translating them into unique phenotypes of the disease in different prion strains is largely unknown. To analyze the mechanistic and structural aspects of the replication of different sCJD prions and specifically the role of sPrPSc, we employed an in-vitro amplification of brain PrPSc with QuIC and sPMCA. Using recombinant human PrP 23–231,129M substrate in QuIC, and Tg mice brains expressing human PrPSc(129M) in the sonication-driven sPMCA, both reactions demonstrated inverse correlation between conversion efficiency and the conformational stability of small protease sensitive (s) oligomers of PrPSc. The observed link between duration of the disease and conversion potency of small oligomers of sPrPSc in individual sCJD cases suggests that these conformers encode the progression rate of the disease in different prion strains.

Results

Amplification index of sCJD PrPSc in sPMCA and QuIC

After four rounds of sPMCA, the Tg(HuPrP,129M) brain homogenate substrate responded to 105-fold diluted sCJD seeds with matching codon 129 polymorphism and Type 1 or Type 2 PrPSc with levels of rPrPSc (PrP 27-30) easily detectable on WBs (Fig. S1 A). The WB mobility of unglycosylated fragments of rPrPSc from individual cases of sCJD matched the original Type 1 or Type 2 seeds (Fig. S1 A). The results extend previous observations on high fidelity replication of sCJD PrPSc using Tg mouse brain expressing homologous human PrPSc [26]. Whether subtle changes in glycosylation pattern also observed previously reflect the changes in the glycosylation of PrPSc in transgenic mice remains to be established [25,26]. The PK treatment of rhuPrPQuIC generated in QuIC resulted in a major PK-resistant fragment with SDS PAGE mobility corresponding to the mass ≈16 kDa (Fig. S1 B). We did not observe differences between masses of peptides generated from rhuPrPQuIC seeded with Type 1 PrPSc(129M) or Type 2 PrPSc(129M). As expected [27], western blot indicated that the 16 kDa fragments, displaying all the epitopes downstream from residue 89, therefore correspond to the sequence 89–231 (Fig. S1 B).

Using CDI, we measured the conversion potency of PrPSc present in sCJD brain homogenates of 20 patients homozygous for methionine in codon 129 and containing either Type 1 (n = 10) or Type 2 (n = 10) PrPSc. Both techniques showed statistically significant inverse correlation of the amplification potency and duration of the sCJD (Fig. 1A and Fig. 1B). The CDI measurements were performed before and after four rounds of sPMCA (Fig. 1A) or after 20 hrs of QuIC (Fig. 1B), at the time points and dilutions that demonstrated maximum differences in amplification between different samples before they reached a plateau [36]. The amplification index (potency) of different seeds is expressed as a ratio between the concentration of the PrPSc in the seed. In each run, the low background levels of PK-resistant PrP generated “de novo” in both sPMCA and QuIC in unseeded samples were subtracted from readings obtained with seeded samples (Fig. S1A and Fig. S2A). The tight correlation between amplification indexes obtained with sPMCA and QuIC in individual samples (Fig. S2B) proved that the observed range of values is highly reproducible with two different techniques. When we separated sCJD samples according WB type of PrPSc, we observed overall higher seeding efficacy of MM1 PrPSc over MM2 PrPSc (Fig. S3). However, the trends were not statistically significant due to the broad range and overlapping amplification values. We concluded from these data that PrPSc present in cases classified as MM1 and MM2 sCJD display a continuum of conversion rates.

The effect of protease treatment of the seeds on amplification rates

Using seeds of Type 1 or Type 2 sCJD PrPSc that were either treated or not with PK before QuIC, we observed up to 10-fold higher seeding potency of the samples that were not treated with PK (Fig. 2). Despite the differences in the response of individual samples, this trend was most prominent with MM1 sCJD PrPSc. With the CDI measurements of recombinant PrP before and after QuIC, we ruled out the possibility that incompletely inactivated PK decreased the concentration of the substrate (Fig. S4). We concluded from these experiments that in QuIC reaction, the total PrPSc is more efficient seed than protease-resistant fragment rPrPSc.

Conformational heterogeneity of MM1 and MM2 sCJD PrPSc

The broad range of amplification indexes within each Type 1 or Type 2 group (Fig. 1, Fig. S3 A, and Fig. S3 B) suggests
conformational heterogeneity beyond that observed with WBs. Therefore, we used the CDI [5,22,37] to determine the strain-dependent conformational range of sCJD PrPSc in patients who were homozygous for methionine in codon 129 of the PRNP gene and demonstrate pure Type 1 or 2 PrPSc on WBs [4]. The definition of sPrPSc as well as rPrPSc is operational and therefore all digestions with proteinase K (PK) were performed at constant protein/enzyme ratio equivalent to 3 IU/ml (100 µg/ml) in 10% brain homogenate containing 1% Sarkosyl for one hour at 37°C. The protocol for PrPSc digestion, validated in previously published experiments, was selected according to the following criteria: 1) complete digestion of PrPC determined with CDI in control samples; 2) complete shift of the bands of PrPSc to PrP 27-30 on WBs; 3) unequivocal WB differentiation of Type 1 and Type 2 rPrPSc in all tested samples [4,38]. The complete digestion of the PrPSc N-terminus with PK was monitored on WBs in all samples.

Using CDI, we measured the concentration and conformational stability of PrPSc in the frontal cortex of individual sCJD patients used in the previous seeding experiments (Table S1). Typical examples of dissociation/unfolding curves before and after PK for MM1 and MM2 PrPSc are shown in Fig. 3A and 3B, respectively. Comparing ten sCJD Type 1 cases, we found a broad range of Gdn HCl1/2 values, from 2.3 to 3.0 M (Table S1 and Fig. 4A). We next investigated the conformational impact of the proteolytic digestion of sPrPSc conformers and the loss of N-terminal residues in rPrPSc. The proteolysis of Type 1 PrPSc(129M) with PK resulted in rPrPSc with invariably increased conformational stability (Fig. 4A).

**Figure 1. Conversion potency of sCJD PrPSc inversely mirrors duration of the sCJD.** Amplification index obtained with (A) sPMCA or with (B) QuIC for MM1 (n = 10) and MM2 (n = 10) sCJD cases. The amplification index is the ratio between the concentration of PrPSc before and after sPMCA or QuIC measured with CDI. The data points and bars are averages ± SEM obtained from three independent conversion experiments, each measured in triplicate with CDI.

doi:10.1371/journal.ppat.1002835.g001
Figure 2. Decreased conversion potency of PrPSc after protease treatment. The sCJD brain homogenates were either (blue bars) untreated or (red bars) treated with 100 μg/ml of PK for 1 hr at 37°C. The PK was blocked with 0.5 mM PMSF, and aprotinin and leupeptin at 5 μg/ml, respectively. Impact of protease treatment of PrPSc on amplification was monitored in QuIC with CDI either in MM1 (n = 3) or MM2 (n = 3) sCJD cases. The bars represent average ± SEM obtained from triplicate measurements. doi:10.1371/journal.ppat.1002835.g002

Comparing ten sCJD Type 2 cases, we found a broad range of Gdn HCl1/2 values, from ~2.6 to ~3.5 M [Table S1 and Fig. 4A]. In contrast to Type 1 sCJD PrPSc, PK treatment of Type 2 PrPSc(129M) uniformly, with one exception, produced rPrPSc with decreased stability (Fig. 4b). The unique case that showed the opposite trend displayed an atypical doublet of 19 and 17 kDa bands on WBs. Taken together, the data demonstrate wide range of unique conformations in both MM1 and MM2 subgroups. The Type 1 rPrPSc(129M) remaining after proteolytic treatment demonstrated higher overall conformational stability than total PrPSc. We observed the opposite effect of PK leading to less stable Type 2 rPrPSc.

To quantify the impact of proteolytic treatment on the conformational stability of PrPSc, we first evaluated the shift in the [Gdn HCl]1/2 values (Table S1). Alternatively, we subtracted the relative fractional change in stability of rPrPSc after PK treatment from the PrPSc values obtained before PK (Table S1, Fig. 3, and Fig. 4C). The resulting differential curves corresponding to the portion of PrPSc (ΔFapp) conformers removed by PK and designated operationally sPrPSc [4]. The differential curves exhibit Gaussian distribution with the peak at the median stability of sPrPSc; the height and integrated peak area is proportional to the relative fraction of PK-digested conformers (ΔFapp). In contrast to simple shift in the [Gdn HCl]1/2 values, the ΔFapp take into account the difference in the slopes of the unfolding curves. Using both shifts in the [Gdn HCl]1/2 and ΔFapp values, the overall stability of Type 1 sPrPSc is lower than that of rPrPSc (Fig. 4C). In contrast, the negative differential curves for Type 2 sPrPSc(129M) and shift in the [Gdn HCl]1/2 values induced by PK both demonstrate that sPrPSc is more stable than rPrPSc in this sCJD group (Fig. 4C). Cumulatively, the data provide important evidence that MM1 and MM2 PrPSc conformers differ in response to proteolytic cleavage, but the observed spread of stability values within each sCJD WB pattern suggests the presence of a broad range of unique PrPSc conformers. Alternatively, data might be also consistent with varying ratios of a small set of conformers that, in themselves, are not unique to each sCJD patient [4,39].

The amplification rates inversely correlate with the stability of sCJD sPrPSc

Comparison of the stability of total PrPSc with the amplification efficacy of individual sCJD PrPSc seeds demonstrate a highly significant inverse correlation for both MM1 (n = 10) and MM2 (n = 10) sCJD samples, and in both sPMCA and QuIC (Fig. 4D). Since this correlation was lost after PK treatment (Fig. 4E) we concluded that sPrPSc fraction of PrPSc must be responsible for the initial correlation. In confirmation, the change in the stability induced by PK correlated to a highly significant degree with the amplification rate (Table S1 and Fig. 4F). Specifically, the samples with less stable sPrPSc (positive shift in [Gdn HCl]1/2 or in Fapp value) showed higher amplification rates (Fig. 4F), and vice versa. We concluded from these experiments that the amplification rates of sCJD PrPSc correlate inversely with the stability of total PrPSc, and that less stable sPrPSc conformers are responsible for this effect. In contrast, the stability of the rPrPSc conformers does not predict the amplification rate of individual sCJD samples, regardless of the WB type.
The relationship between prion size, protease sensitivity, and conversion efficacy of sCJD PrPSc

To investigate the impact of prion particle size on protease sensitivity and amplification, we separated sCJD prion particles according to sedimentation velocity using high-speed centrifugation in sucrose gradient. The sCJD prions present in brain homogenates of twelve sCJD patients with MM1 (n = 6) or MM2 (n = 6) PRNP gene polymorphism and WB pattern of PrPSc were separated in 10–45% sucrose gradient and collected fractions were analyzed by WBs, CDI, and QuIC. The PrPC in platelets of healthy donors as well as in brain tissue of patients that had other neurologic disorders remained in the top portion of the tube, as expected for monomers or possible dimers of PrPC (Fig. S6). Similarly, PrPC present in MM1 and MM2 sCJD cases remained in the upper portions of the tubes (Fig. 5A, Fig. 5B, and Fig. 5C). In contrast, the MM1 (n = 6) and MM2 (n = 6) PrPSc sedimented into sucrose with a broad range of densities, and a variable fraction remained floating (Fig. 5A, Fig. 5B, and Fig. 5C). The peak sedimentation velocity of MM1 PrPSc is reproducibly slower than that of MM2 PrPSc (Fig. S6). Similarly, PrPSc present in MM1 and MM2 sCJD cases remained in the upper portions of the tubes (Fig. 5A, Fig. 5B, and Fig. 5C). The sedimentation velocity of the majority of MM2 sCJD PrPSc conformers induced by PK in individual sCJD samples (filled red circles) Type 1 PrPSc(129M), and (filled blue squares) Type 2 PrPSc(129M). The (D) inverse relationship between stability of total PrPSc and amplification index; (E) no correlation between stability of PrPSc and amplification index; and (F) direct correlation between PK-induced change in the stability of PrPSc (Δ Fapp) and amplification index. The stability of prion and conversion potency of PrPSc was determined by CDI and expressed as Gdn HCl1/2 or stability change (Δ Fapp) induced by PK. Each symbol represents an average of triplicate experiment followed by triplicate measurement ± SEM with CDI.

doi:10.1371/journal.ppat.1002835.g004

Figure 4. Relationship between conversion potency and the conformational stability of PrPSc, rPrPSc, and sPrPSc in MM1 (n = 10) and MM2 (n = 10) sCJD cases. The (A) conformational stability of MM1 PrPSc before (red diamonds) and after (red circles) PK digestion; the (B) conformational stability of MM2 PrPSc before (blue triangles) and after (blue squares) PK digestion; and the (C) fractional change in stability of PrPSc conformers induced by PK in individual sCJD samples (filled red circles) Type 1 PrPSc(129M), and (filled blue squares) Type 2 PrPSc(129M). The (D) inverse relationship between stability of total PrPSc and amplification index; (E) no correlation between stability of PrPSc and amplification index; and (F) direct correlation between PK-induced change in the stability of PrPSc (Δ Fapp) and amplification index. The stability of prion and conversion potency of PrPSc was determined by CDI and expressed as Gdn HCl1/2 or stability change (Δ Fapp) induced by PK. Each symbol represents an average of triplicate experiment followed by triplicate measurement ± SEM with CDI.

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The amplification index obtained from each fraction with QuIC showed the highest conversion potency for the fractions with S values from 18 to 30 and significantly lower amplification potency of PrPSc with increasing size of the aggregates in MM1 (n = 6) as well as MM2 (n = 6) sCJD cases (Fig. 5D and Fig. 5E). The PK resistance profile of both MM1 and MM2 PrPSc obtained with CDI indicates a statistically significant (MM1: P < 0.001; MM2: P = 0.034) trend toward higher PK sensitivity of the fractions containing small oligomers of PrPSc in comparison to the bottom
Figure 5. Sedimentation velocity, amplification index, and protease sensitivity of PrPSc present in frontal cortex of patients with sCJD Type MM1 (n = 6) and Type MM2 (n = 6) and fractionated by ultracentrifugation in sucrose gradient. Fractions were collected from the bottom of the tubes and PK-treated or untreated samples were analyzed for PrP by (A) WB with biotinylated primary antibody 3F4 and secondary Streptavidin-Peroxidase complex. The WBs are typical examples of Other Neurological Disease (OND), Type 1 (sCJD MM1), and Type 2 sCJD (sCJD MM2) homozygous for methionine in codon 129 of PRNP gene. The cumulative plots of concentration of PrPSc (green bars), total PrPSc (blue bars), and rPrPSc (red bars) in sucrose fractions was determined with CDI before or after PK treatment in six cases of (B) sCJD type MM1 and six cases of (C) Type MM2. The bars represent average ± SEM (n = 6); CDI was performed in each sCJD case in triplicate. The amplification index (red squares) determined with QuIC and relative concentration of sPrPSc (blue circles) measured by CDI and QuIC in (D) MM1 (n = 6) and (E) MM2 (n = 6) sCJD PrPSc separated in sucrose gradient. The points and bars are average ± SEM; both QuIC and CDI were performed in triplicate for each sCJD case sample. The asterisk in WBs indicates the band of PK cross reacting with primary antibody. The molecular mass of the markers is in kDa.

doi:10.1371/journal.ppat.1002835.g005
that the most potent seeds of sCJD PrPSc have a mass 0.45–

 exceed 4%.

[41], the error of our estimates due to the lipid content does not
detergent solubilization and centrifugation in sucrose gradient

However, assuming a 2:1 molar ratio of PrP Sc monomer to

estimates could come from lipids bound in PrP Sc aggregates.

influenced sedimentation velocity by

sphingolipids and cholesterol remaining in PrPSc aggregates after

prions after protease treatment, the stability and sedimentation

velocities of mm2 sCJD (Fig. 5A). In CDI, the impact of
differential mAb binding is corrected by separate calibration with

HuPrP(29-231) for PK-untreated samples and with HuPrP(90-231)

for PK-treated samples [4].

When we expressed the levels of small oligomers of PrPSc with

the highest seeding potency that eluted between 2.4–3.2 ml of

sucrose gradient as a percentage of total PrPSc, we observed an

inverse correlation with the duration of the sCJD (Fig. 6).

Cumulatively, the diminished amplification potency of the sCJD

prions after protease treatment, the stability and sedimentation

velocity data indicate that the highest seeding potential in QuIC

have relatively PK-sensitive small aggregates of PrPSc. We estimate

that the most potent seeds of sCJD PrPSc have a mass 0.45–

1.80 × 10^6 Da and are composed of 20–78 monomers of PrPSc.

The control experiments with standard proteins performed in the

presence or absence of Sarkosyl indicate that Sarkosyl binding

influenced sedimentation velocity by ~8%. Additional error in our

estimates could come from lipids bound in PrPSc aggregates.

However, assuming a 2:1 molar ratio of PrPSc monomer to

sphingolipids and cholesterol remaining in PrPSc aggregates after

detergent solubilization and centrifugation in sucrose gradient

[41], the error of our estimates due to the lipid content does not

exceed 4%.

Discussion

In this study, we used novel conformational methods derived

from CDI in tandem with two different amplification techniques

for PrPSc to determine the spectrum of sCJD prions and to

investigate conformational features that control conversion reac-
tion. Surprisingly, we identified within each clinical and pathologic
category of sCJD an array of PrPSc structures that differ in

protease-sensitivity, conformational stability, and conversion

potency. Each of these features offers evidence of a distinct prion
strain and suggests that the extraordinary clinicopathologic
variability of sCJD stems from the broad range of prions causing
the disease and imply stochastic origin or conformational evolution
during prion propagation in the brain. Our data on human brain
sCJD prions indicate an intimate relationship between the
conformation, size, and conversion potency of the small prote-

ase-sensitive oligomers of PrPSc.

Sources of phenotypic and molecular diversity of sCJD

The distinct conversion efficacies we observed with prion seeds

obtained from different cases of MM1 and MM2 sCJD were

highly reproducible with both QuIC and sPMCA techniques (Fig.

S2). Cumulatively, the data demonstrate that the conversion

potency of PrPSc in individual isolates of sCJD is inversely related
to the duration of the disease. Because the length of the duration of

a clinically pronounced prion disease is a function of the

incubation time, this finding accords with the bioassay data that

show a broad spectrum of transmission rates and incubation times

of sCJD prions in transgenic mice expressing human PrP^P(129M)
or human/mouse PrPSc chimeras [5,6,42]. Therefore, our

demonstration that disease duration in sCJD patients mirrored

the conversion efficacy of PrPSc suggests that both sPMCA and

QuIC reproduce important kinetic aspects of prion replication

in vivo, though infectivity properties of both reaction products are

yet to be tested. The distinctness in replication efficacy is a principal

hallmark of prion strains; it leads to different incubation times and

progression rates of the disease in bioassays [1]. The statistically

insignificant difference between Type MM1 PrPSc and MM2

PrPSc and broad distribution of values within each group suggests

that a continuum of sCJD prions are causing the disease.

The striking range of conformational stability values of PrPSc

found with CDI before or after PK in sCJD patients homozygous

for codon 129 pmyrophism of the PRNP gene and showing either

pure Type 1 or Type 2 WB pattern (Fig. 4A and Fig. 4B) by far

exceeds the variability expected from the sampling or method itself

[4]. The observed differences in stability offer evidence of a broad

spectrum of different conformations of PrPSc present in vivo

in MM1 and in MM2 sCJD patients [4,43]. The increased frequency

of exposed epitopes and decreased stability in Type 2 PrPSc after

PK treatment [4] are counterintuitive and may indicate one of

three possibilities: that the PK sensitivity is not an obligatory

measure of protein stability and rPrPSc may be in some prion

strains less stable than sPrPSc; that removal of the N-terminus

from PrPSc resulted in less stable conformation with more exposed

108–112 epitopes; or that the ligand protecting the 108–112

epitope and stabilizing the PrPSc was removed by PK. Whether

the epitopes’ hindrance in undigested PrPSc is the result of lipid,
glycosaminoglycan, nucleic acid, or protein binding to the

conformers unique to the MM2 sCJD PrPSc remains to be

established. Since sCJD cases with Type 2 PrPSc(129M) have

generally extended disease durations, the molecular mechanism

underlying this effect calls for detailed investigation. Cumulatively,

the conversion and stability data support the conclusion that each

MM1 or MM2 category of sCJD prions causes an accumulation of

a broad range of distinct conformers of PrPSc in the individual

sCJD brains. Whether individual sCJD case is caused by a single

conformer having unique replication efficacy or a varying ratios of

a small set of conformers that, in themselves, are not unique to

each sCJD patient remains to be seen. Consequently, since all

these phenomena characterize prion strains, the data suggest that

\[\text{Figure 6. The inverse relationship between proportion of small}
\text{oligomers of PrPSc and duration of sCJD. The levels of small}
\text{oligomers of PrPSc with the highest seeding potency that eluted at 2.4–}
\text{3.2 ml of sucrose gradient (Fig. 5D and Fig. 5E) were expressed as a}
\text{percentage of total PrPSc in (circle) MM1 (n = 6) and (square) MM2}
\text{(n = 6) sCJD cases. Each symbol represents an average ± SEM.}
\text{doi:10.1371/journal.ppat.1002835.g006} \]
The impact of levels and stability of protease-sensitive conformers of PrPSc on conversion potency of human prions

Considerable data demonstrate that sPrPSc replicates in vivo and in vitro as an invariant and major fraction of PrPSc and that the proteolytic sensitivity of PrPSc can reliably differentiate various prion strains [5,22,44,45]. Accumulation of sPrPSc precedes protease-resistant product (rPrPSc) in prion infection [46]; and up to 90% of PrPSc accumulating in CJD brains consists of sPrPSc [5,47]. It is noteworthy that amyloid fibrils produced in vitro from recombinant mouse PrP generated prions composed exclusively of sPrPSc upon inoculation into wild mice [19].

Previously we found that levels of sPrPSc varied with the incubation time of the disease [22] and we hypothesized that the molecular mechanism responsible for this phenomenon was related to the replication or clearance of prions [5,22]. Subsequent experiments in yeast indicated that replication rate of prions may be an inverse function of the stability of misfolded protein [48]. The hypothesis based on these experiments posits that the less stable prions replicate faster by exposing more available sites for the growth of the aggregates. Although more recent experiments with rodent and synthetic prions showed the correlation between shorter incubation time of prions that converted to protease-sensitive isoforms at a lower denaturant concentrations. The replication rate of mammalian prions was never measured and the observed effect could be equally well explained by different clearance rates of distinct prions [49,50].

The experiments presented here determined the impact of the overall stability of sCJD PrPSc, rPrPSc, and sPrPSc on the seeding potency of sCJD prions with QuIC and sPMCA. Both methods revealed that the stability of total PrPSc inversely correlated with the amplification index of sCJD PrPSc. Moreover, when sPrPSc proved less stable than rPrPSc, the difference in stability correlated with more efficient amplification. Conversely, when sPrP con- formers proved more stable than rPrPSc, we observed the opposite effect—less accumulated PrPSc in both sPMCA and QuIC (Fig. 4). The absent clearance in both methods lead to the conclusion that lower stability of PrPSc conformers, and specifically sPrPSc conformers, is an important determinant of the conversion rate and that these conformers likely play an important role in the incubation time and progression of the diseases in vivo.

The relationship between conversion efficacy and prion size

Although prion diseases were originally characterized by deposits of protease-resistant PrPSc, often with large amyloid fibrils, there is growing recognition that protease resistance and amyloid fibrils do not constitute obligatory factors in the pathogenesis of these diseases [5,11,22]. These findings have accordingly raised certain questions; specifically, whether the amyloid fibrils cause the disease; and whether smaller non-amyloid oligomers of PrPSc comprise the initial steps in prion replication, or are related to the alternative misfolding pathway [5,22,44]. Subsequent evaluation of the converting activity and of the size of aggregates dissociated from purified Syrian hamster PrPSc with ionic detergent sodium n-undecyl sulphate suggested that the maximum seeding activity is associated with relatively small aggregates dissociated from longer fibrils [23]. However, it remained unclear whether such highly potent small oligomers exist also in vivo.

In this research, we investigated whether small aggregates of PrPSc with high seeding potency exist in the brains of patients with sCJD. The broad range of sedimentation velocities observed with ultracentrifugation of sCJD brain homogenates in sucrose gradient indicate that sCJD PrPSc proteins exist in the continuum of aggregates composed from <20 to >600 PrPSc molecules. Surprisingly, small oligomers of human PrPSc, with masses equivalent to 20-78 PrPSc molecules, are the most efficient initiators of PrPSc conversion, and the seeding efficacy of sCJD prions actually decreased with the size of the aggregates. Interestingly, the variable fraction of PrPSc remained with PrPSc in the upper layers of sucrose gradient. This phenomenon may suggest even smaller oligomers or, alternatively, association with detergent-insoluble lipids present in cholesterol-rich domains (caveolae) of cellular membranes. This association would place both cellular and pathogenic forms of the prion protein into the same compartment and thus support the hypothesis that PrPSc formation occurs within caveolae [40].

The strikingly high sedimentation velocity of MM2 PrPSc in contrast with the lower sedimentation velocity of MM1 PrPSc indicates that MM2 PrPSc forms much larger aggregates and concurs with the pattern of large coarse deposits of Type 2 PrPSc observed with the brain immunohistochemistry in situ, in contrast to the fine (punctate, “synaptic”) appearance of the immunoreactivity associated with Type 1 PrPSc [10]. Cumulatively, these data suggest that the distinct quaternary structure or packing of the monomers of PrPSc may be responsible for the different peptide fragmentation pattern with predominantly 19 kDa fragments of MM2 rPrPSc and 21 kDa in MM1 rPrPSc after PK treatment.

Materials and Methods

Ethics statement

All procedures were performed under protocols approved by the Institutional Review Board at Case Western Reserve University. In all cases, written informed consent for research was obtained from patients or legal guardians and the material used had appropriate ethical approval for use in this project. All patients’ data and samples were coded and handled according to NIH guidelines to protect patients’ identities.

Patients and clinical evaluations

We selected 20 representative subjects from a group of 340 patients with definitive diagnosis of sCJD. The criteria for inclusion were (1) availability of clinical diagnosis of CJD according to WHO criteria [51,52] and clearly determined and dated initial symptoms upon neurologic examination to ascertain the disease duration; (2) methionine homozygous at codon 129 of the human prion protein (PrP) gene (PRNP); (3) unequivocal classification as pure Type 1 or Type 2 sCJD according to WB pattern; (4) unequivocal classification of pathology as definite Type 1 or 2 at the National Prion Disease Pathology Surveillance Center (NPDPSC) in Cleveland, Ohio; (5) demographic data distribution within 95% confidence interval of the whole group, resulting in no difference between selected cases and the whole group in any of the statistically followed parameters.

Retrospective charts review was carried out for all subjects, with particular attention to the documented initial cardinal clinical signs of sCJD such as cognitive impairment, ataxia, and myoclonus [51,52]. We also reviewed the findings on electroencephalography, brain magnetic resonance imaging, and CSF markers when available.
Brain samples and PRNP gene sequencing

All Type 1–2 patients or uncertain cases were excluded from this study. DNA was extracted from frozen brain tissues in all cases, and genotypic analysis of the PRNP coding region was performed as described [5,53]. On the basis of diagnostic pathology, immunohistochemistry, and western blot (WB) examination of 2 or 3 brain regions (including frontal, occipital and cerebellum cortices) with mAb 3F4, the pathogenic PrPSc was classified as (1) Type 1 PrPSc(129M) (n = 10) and (2) Type 2 PrPSc(129M, n = 10). Patients lacked pathogenic mutations in the PRNP and had no history of familial diseases or known exposure to prion agents. These cases contained pure Type 1 PrPSc(129M) or Type 2 PrPSc(129M).

Western blots

Both PK-treated and untreated samples were diluted 9-fold in 1× Laemmli Buffer (Bio-Rad, Hercules, California) containing 5% (v/v) beta-mercaptoethanol (ME) and final 115 mM Tris-HCl, pH 6.8. Samples were heated for 5 min at 100°C and ~2 ng of PrP per lane was loaded onto 1 mm 15% Polyacrylamide Tris-GdnHCl gel. Membranes were blocked with 2% (w/v) BSA in TBS containing 0.1% of Tween 20 (v/v) and 0.05% (v/v) Kathon CG/IPC (Sigma, St. Louis, Missouri). The PVDF membranes were developed with 0.05 ug/ml of biotinylated mAb 3F4 (Govance, Princeton, New Jersey) followed by 0.0175 ug/ml Streptavidin-Peroxidase conjugate (Fisher Scientific, Pittsburgh, Pennsylvania) or with ascitic fluid containing mAb 3F4 (kindly supplied by Richard Kacskak) diluted 1:20,000 followed by Peroxidase-labeled sheep anti-mouse IgG Ab (Amersham, Piscataway, New Jersey) and diluted 1:3000. The membranes were developed with the ECL Plus detection system (Amersham) and exposed to Kodak BioMax MR Films (Fisher Scientific) or Kodak BioMax XAR Films (Fisher Scientific).

Conformation and Seeding Potency of sCJD PrPSc by CDI

The CDI for human PrP was performed as described previously [4,5,34], with several modifications. First, we used white Lumitrac 600 High Binding Plates (E&K Scientific, Santa Clara, California) coated with mAb 8H4 (epitope 175–185) [55] in 200 mM NaH2PO4 containing 0.03% (w/v) NaN3, pH 7.5. Second, aliquots of 20 µl from each fraction containing 0.007% (v/v) of Patent Blue V (Sigma) were directly loaded into wells of white strip plates prefilled with 200 µl of Assay Buffer (Perkin Elmer, Waltham, Massachusetts). Finally, the captured PrP was detected by a europium-conjugated [22] anti-PrP mAb 3F4 (epitope 107–112) [56] and the time-resolved fluorescence (TRF) signals were measured by the multi-mode microplate reader PHERAstar Plus (BMG LabTech, Durham, North Carolina). The recHuPrP(90-231,129M) and PrP(23-231,129M) were used as a calibrator in the CDI was prepared and purified as described previously [57]. The initial concentration of recombinant human PrP(23-231) and PrP(90-231) was calculated from absorbance at 280 nm and molar extinction coefficient 5630 M\(^{-1}\) cm\(^{-1}\) and 21640 M\(^{-1}\) cm\(^{-1}\), respectively. The purified recombinant proteins were dissolved in 4 M GdnHCl and 50% Stabilcoat (SurModics, Eden Prairie, Minnesota), and stored at ~8°C. The concentration of PrP was calculated from the CDI signal of denatured samples using calibration curve prepared with either recHuPrP(23-231) for samples containing full length PrPSc or recPrP(90-231) for samples containing truncated rPrPSc (PrP 27-30) after proteinase-K treatment. This separate calibration was necessary due to the ~3.5-fold lower affinity of mAb 3F4 with full-length human PrP(23-231,129M) compared to PrP(90-231,129M) [4].

Monitoring dissociation and unfolding of PrPSc by CDI

The denaturation of human PrPSc was performed as described previously [4,22], with several modifications. Frozen aliquots of PrPSc were thawed, sonicated 3 x 5 s at 60% power with Sonicator 4000 (Qonica, Newtown, Connecticut), and the concentration was adjusted to constant ~50 ng/ml of PrPSc. The 15 µl aliquots in 15 tubes were treated with increasing concentrations of 8 M GdnHCl containing 0.007% (v/v) Patent Blue V (Sigma, St. Louis, Missouri) in 0.25 M or 0.5 M increments. After 30 min incubation at room temperature, individual samples were rapidly diluted with Assay Buffer (Perkin Elmer, Waltham, Massachusetts) containing diminishing concentrations of 8 M GdnHCl, so that the final concentration in all samples was 0.411 M. Each individual aliquot coating (Fisher Scientific, Pittsburgh, Pennsylvania) or with ascitic fluid containing mAb 3F4 (kindly supplied by Richard Kacskak) diluted 1:20,000 followed by Peroxidase-labeled sheep anti-mouse IgG Ab (Amersham, Piscataway, New Jersey) and diluted 1:3000. The membranes were developed with the ECL Plus detection system (Amersham) and exposed to Kodak BioMax MR Films (Fisher Scientific) or Kodak BioMax XAR Films (Fisher Scientific).

The raw TRF signal was converted into the apparent fractional change of unfolding (Fapp) as follows [4]:

\[
F_{\text{app}} = F_0 + \left( \frac{F_{\text{max}} - F_0}{1 + e^{-\left( c_{1/2} - c \right)}} \right)
\]

The apparent fractional change (F\(\text{app}\)) in the TRF signal is the function of Gdn HCl concentration(c), c\(_{1/2}\) is the concentration of
Gdn HCl at which 50% of PrPSc is dissociated/unfolded and r is the slope constant. To determine the impact of protease treatment on the conformational stability of PrPSc, the values of fractional change after PK were subtracted from \( \Delta F_{app} \) values obtained before PK (\( \Delta F_{app} = F_0 - F_{PK} \)) and then fitted with a Gaussian model to estimate the proportion and average stability of sPrPSc conformers (Equation 2):

\[
\Delta F_{app} = F_0 + A \left( e^{-\left(\frac{r-t}{\sigma}\right)^2}\right)
\]

In this model, the PK-induced fractional change is \( \Delta F_{app} \), \( F_0 \) is fractional change at 0 concentration of Gdn HCl, and \( \sigma \) is the Gdn HCl concentration at the maximum height A of the peak [4].

Quaking-Induced Conversion (QuIC)

The QuIC was performed as described [27] with the following modifications. The rhuPrP(90-231,129M) used as a substrate in QuIC was expressed, purified, and refolded to \( \alpha \)-helical conformation as described previously [57]. The initial concentration of recombinant human PrP(23-231) was calculated from absorbance at 280 nm and molar extinction coefficient 56650 M\(^{-1}\) cm\(^{-1}\). The stock of rhuPrP(23-231) in 10 mM Sodium Acetate buffer, pH 4.0, was treated with 12 mM HCl at 1:3.9 (rhuHuPrP : HCl, v/v) ratio for 7.5 min and immediately diluted to final 0.1 mg/ml into the reaction buffer composed of 20 mM HCl, 0.1% Triton X-100, and 1:5000 (v/v) N\(_2\) (Invitrogen, Carlsbad, California). The QuIC was performed with final volume 100 \( \mu \)l per well in sterile V-bottom, low-binding polypropylene 96-well plate (VWR, Avon, Ohio) set for 1 min shaking at 1400 rpm.

To each well containing 100 \( \mu \)l of QuIC reaction buffer was added 50 \( \mu \)l of PBS, pH 6.9, containing 3% (w/v) Sarkosyl and Proteinase K (PK; Amresco, Solon, Ohio) to obtain the final 10\(^{-4}\) dilution of sCJD prions, and the plates were sealed with sterile AxyMat Silicone Sealing Mat (VWR, Avon, Ohio). The QuIC was performed in samples seeded with sCJD PrP(23-231, 129M) substrate (\( \alpha \)-PrPSc). The QuIC was expressed, purified, and refolded to \( \alpha \)-helical conformation as described previously [57]. The initial concentration of rhuPrPSc was calculated from absorbance at 280 nm and molar extinction coefficient 56650 M\(^{-1}\) cm\(^{-1}\) at 280 nm. The QuIC was performed with final volume 100 \( \mu \)l in wells of a 96-well plate (VWR, Avon, Ohio) on a microshaker (Misonix, Farmingdale, New York) set for 1 min shaking at 1400 rpm, and immersed in the water of the sonicator bath.

Sucrose gradient ultracentrifugation

The 400 \( \mu \)l aliquots of 10% brain homogenate in PBS, pH 7.4, containing 2% Sarkosyl were clarified by centrifugation at 500 × g for 5 min and carefully layered onto the top of the 10–45% sucrose gradient. The sucrose gradient was prepared in PBS, pH 7.4, containing 1% Sarkosyl in Thinwall Polyallomer (18 x 60 mm) tubes (Beckman, Palo Alto, California). The ultracentrifugation was performed at 50,000 rpm for 73 min at 5°C in Optima TL ultracentrifuge (Beckman, Palo Alto, California) equipped with Beckman SW 55 Ti rotor. These conditions correspond to the adjusted proportionality constant \( k = 58.7 \) and angular velocity \( \omega = 5236 \) rad/s. Observed sedimentation coefficients \( s_{obs} \) were calculated from formula \( s_{obs} = k/\omega^2 \) where \( t \) is the centrifugation time. The \( s_{20w} \) values for given angular velocity, prion particle density 1.35 g/ml [61], and sucrose density and viscosity were calculated as described [62,63]. The second approach to estimate the S values for the upper layers of sucrose gradient was calibration with bovine serum albumin (BSA, S = 4.4, MW = 67 kDa), alcohol dehydrogenase (ADH, S = 7.9, MW = 150 kDa), thyroglobulin monomer (TG, S = 12.0, MW = 335 kDa), and apofercin (AF, S = 17.0, MW = 143 kDa) [63]. After the centrifugation, the 200 or 400 \( \mu \)l fractions of gradients were collected from the bottom of the tube.

Statistical analysis

We investigated the effect of the following demographic and laboratory variables on survival: sex; age at onset; duration of the disease; electrophoretic Type of PrP 27-30; and the concentration and stability of PrPSc in Gdn HCl before and after PK treatment [22]. In the comparison of different groups, P values were calculated using Anova. Cumulative survival curves were constructed by the Kaplan–Meier method, both overall and by stratifying for each of the above variables. Comparisons of survival curves among groups were carried out by the log rank (Mantel–Cox) and generalized Wilcoxon test. All the statistical analyses were performed using SPSS 19 software (SPSS Inc., Chicago, Illinois).

Supporting Information

Figure S1 A typical amplification of sCJD prions in sPMCA using brain homogenate of Tg mice expressing human PrP(129M) and with QuIC using recombinant human PrP(23-231, 129M) substrate. (A) Sonication-driven sPMCA with brain PrP\(^{\alpha}\) substrate preserves the differences in mobility of unglycosylated PrPSc in three MM1 and three MM2 sCJD PrP\(^{\alpha}\) after four rounds of amplification and final dilution of brain sCJD PrP\(^{\alpha}\) 10\(^{4}\)-fold. Asterisk signifies residual full length PrP after PK treatment. (B) 16 kDa protease-resistant fragments of rhuPrP(23-231) detected after QuIC by WBs developed with monoclonal antibody 12B2 (epitope residues 89–93) (21) or 3F4 (epitope residues 107–112) (9). The S20w values for given angular velocity, prion particle density 1.35 g/ml [61], and sucrose density and viscosity were calculated as described [62,63]. After the centrifugation, the 200 or 400 \( \mu \)l fractions of gradients were collected from the bottom of the tube.

Figure S2 The time course of QuIC reaction and comparison with sPMCA. (A) The typical results of QuIC seeded with different sCJD prions and low background levels of de novo PrP. (B) The amplification of sCJD PrP\(^{\alpha}\) after four rounds of sPMCA (n = 20) correlates to a highly significant degree with QuIC (n = 20). The
amplification index is the ratio between the concentration of PrPSc before and after PMCA measured with CDI. The data points are averages of three PMCA experiments, each measured in triplicate with CDI. (TIF)

Figure S3 Continuum of amplification indexes of PrPSc recorded with both sPMCA and QuIC in MM1 and MM2 sCJD. (A) Amplification index obtained with sPMCA for (red circles) MM1 (n = 10) and (blue squares) MM2 (n = 10) sCJD cases. (B) Amplification index obtained with QuIC for (red circles) MM1 (n = 10) and MM2 (n = 10) sCJD. In both sPMCA and QuIC, the differences observed between MM1 and MM2 samples are not statistically significant. The amplification index was determined as described in legend for Figure 1 and the data points are averages ± SEM obtained from three independent PMCA experiments, each measured in triplicate with CDI. (TIF)

Figure S4 The CDI demonstrated the same end point concentrations of recombinant PrP substrate in samples with PK treated or untreated seeds at the end of the QuIC reaction. The total (substrate+seed) PrP concentration was measured with CDI in duplicate in six samples at the end of QuIC reaction. (TIF)

Figure S5 Calibration of sucrose gradient ultracentrifugation with standard proteins. The (A) elution profile and (B) calibration plot for estimating S20,w and mass. The calibrants were bovine serum albumin (BSA, S = 4.4, MW = 67 kDa), alcohol dehydrogenase (ADH, S = 7.9, MW = 150 kDa), thyroglobulin monomer (TG, S = 12.0, MW = 335 kDa), and apolipoprotein (AF, S = 17.0, MW = 443 kDa). (20) The protein content in each fraction was determined with BCA protein assay (Pierce, Rockford, Illinois.). (TIF)

Figure S6Sucrose gradient ultracentrifugation of control samples containing only PrPC. The (A) PrPSc in the human platelets isolated from healthy donors and (B) in a control brain of the patient with other than prion disease (OND). The distribution of PrPSc (green bars), total PrPSc (blue bars), and rPrPSc (red bars) in sucrose fractions was determined with and without PK treatment by CDI. The PrPSc and rPrPSc values oscillating around zero were used to establish the cutoff and baseline sensitivity limit of CDI in each fraction. The bars represent average ± SEM from CDI performed in triplicate. (TIF)

Figure S7 Fractionation by ultracentrifugation in sucrose gradient and protease sensitivity of samples taken from frontal cortex of individual (left column) MM1 (n = 6) and (right column) MM2 sCJD (n = 6) sCJD cases. The distribution of PrPSc (green bars), total PrPSc (blue bars), and rPrPSc (red bars) in sucrose fractions was determined with and without PK treatment by CDI. The bars represent average ± SEM from CDI performed in triplicate. (TIF)

Table S1 Descriptive statistics of the data and demographics of sCJD cases. (DOC)

Acknowledgments

The authors thank to Ms. Diane Kofsky for her invaluable technical help.

Author Contributions

Conceived and designed the experiments: WKS JGS. Performed the experiments: CK TH YC WC MC. Analyzed the data: WKS JGS. Contributed reagents/materials/analysis tools: KS JB M-SS QK GCT. Wrote the paper: JGS WKS.

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