Protease-Sensitive Conformers in Broad Spectrum of Distinct PrPSc Structures in Sporadic Creutzfeldt-Jakob Disease Are Indicator of Progression Rate

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Abstract

The origin, range, and structure of prions causing the most common human prion disease, sporadic Creutzfeldt-Jakob disease (sCJD), are largely unknown. To investigate the molecular mechanism responsible for the broad phenotypic variability of sCJD, we analyzed the conformational characteristics of protease-sensitive and protease-resistant fractions of the pathogenic prion protein (PrPSc) using novel conformational methods derived from a conformation-dependent immunoassay. In 46 brains of patients homozygous for polymorphisms in the PRNP gene and exhibiting either Type 1 or Type 2 western blot pattern of the PrPSc, we identified an extensive array of PrPSc structures that differ in protease sensitivity, display of critical domains, and conformational stability. Surprisingly, in sCJD cases homozygous for methionine or valine at codon 129 of the PRNP gene, the concentration and stability of protease-sensitive conformers of PrPSc correlate with progression rate of the disease. These data indicate that sCJD brains exhibit a wide spectrum of PrPSc structural states, and accordingly argue for a broad spectrum of prion strains coding for different phenotypes. The link between disease duration, levels, and stability of protease-sensitive conformers of PrPSc suggests that these conformers play an important role in the pathogenesis of sCJD.

Introduction

Prions cause a group of fatal and rapidly progressing neurodegenerative diseases, originally described as transmissible spongiform encephalopathies (TSEs) [1,2]. The most common of these diseases is sporadic Creutzfeldt-Jakob disease (sCJD), which accounts for ~85% of all CJD cases worldwide [3]. Although 40 years ago sCJD was shown to be transmissible to nonhuman primates [4], its pathogenesis remains enigmatic.

Most researchers today believe that all prion diseases are caused by the accumulation of an aberrantly folded isoform, termed PrPSc, of the prion protein PrP [5]. Having a basic amino acid composition and an unstructured N-terminus, PrP can assume at least two conformations: (1) native, α-helix-rich PrP0 and (2) disease-causing, β-sheet-rich PrPSc [6–8]. The latter represents a misfolded isoform of the normal cellular prion protein PrPc, which is host-encoded by the chromosomal gene PRNP and expressed at different levels in mammalian cells [9]. Yet despite the impressive progress that has been made in understanding the molecular basis of prion diseases, the molecular mechanism of initial misfolding and the high-fidelity replication of the pathogenic conformation of PrPSc in vivo both remain elusive [2,10–12].

Many lines of evidence from experiments with laboratory prion strains support the view that the phenotype of the disease—its distinctive incubation time, clinical features, and brain pathology—is enciphered in the strain-specific conformation of PrPSc [13–17]. Although remarkable progress has been made in understanding the structure of laboratory strains of rodent prions [2,10,18–20], knowledge of the molecular basis of human prion diseases has lagged behind. Researchers generally agree that the genotype at codon 129 of the chromosomal gene PRNP underlies susceptibility to these diseases and, to some degree, their phenotype [21]. However, in contrast to the experiments with laboratory rodent prion strains, in which the digestion of brain PrPSc with proteolytic enzyme proteinase K (PK) consistently results in a single protease-resistant domain with mass ~19 kDa, the outcome in sCJD is more complex. Distinctive glycosylation patterns and up to four PK-resistant fragments of the pathogenic prion protein (rPrPSc) found in sCJD brains are easily distinguishable on western blot (WB) [14,21–25]. The WB findings together with PRNP gene polymorphism led Parchi, Gambetti, and colleagues to posit a clinicopathological classification of sCJD into five or six subtypes; notably, the WB characteristics of PrPSc breed true upon transmission to susceptible transgenic mice [14,21,22]. An alternative classification of the PrPSc types and their pairing with CJD phenotypes has been proposed by Collinge and collaborators [23,24,26,27]. This classification differs from the previous one in two major aspects: First, it recognizes three (not...
Author Summary

Sporadic Creutzfeldt-Jakob disease (sCJD) is the most common human prion disease worldwide. This neurodegenerative disease, which is transmissible and invariably fatal, is characterized by the accumulation of an abnormally folded isoform (PrPSc) of a host-encoded protein (PrP0), predominantly in the brain. Most researchers believe that PrPSc is the infectious agent and five or six subtypes of sCJD have been identified. Whether or not these subtypes represent distinct strains of sCJD prions is debated in the context of the extraordinary variability of sCJD phenotypes, frequent co-occurrence of different PrPSc fragments in the same brain, and the fact that up to 90% of protease-sensitive PrPSc eludes the conventional analysis because it is destroyed by protease treatment. Using novel conformational methods, we identified within each clinical and pathological category an array of PrPSc structures that differ in protease-sensitivity, display of critical domains, and conformational stability. Each of these features offers evidence of a distinct conformation. The link between the rate at which the disease progresses, on the one hand, and the concentration and stability of protease-sensitive conformers of PrPSc on the other, suggests that these conformers play an important role in how the disease originates and progresses.

two) PrPSc electrophoretic mobilities; and second, it identifies also PrPSc isoforms with different ratios of the three PrP glycoforms [26]. Although the disease phenotypes of patients with sCJD are remarkably heterogeneous, 21 kDa fragments of unglycosylated PrPSc (Type 1) frequently differ from the phenotypes associated with the 19 kDa fragments of unglycosylated PrPSc (Type 2) [14,21,22,28]. Cumulatively these findings argue that the PrPSc type represents yet an additional major modifier in human prion diseases; accordingly, WB-based clinicopathologic classifications became an important tool in studies of prion pathogenesis in human brains and in transgenic mice models [14,26]. Now, inasmuch as two distinct PK cleavage sites in PrPSc Types 1 and 2 most likely stem from distinct conformations, some investigators contend that PrPSc Types 1 and 2 code distinct prion strains [14,23,28,29]. However, the heterogeneity of sCJD, along with a growing number of studies including bioassays, all suggest that the range of prions causing sCJD exceeds the number of categories recognized within the current WB-based clinicopathologic schemes [30–32]. Additionally, recent findings revealed the co-occurrence of PrPSc Types 1 and 2 in up to 44% of sCJD cases and thus created a conundrum [33–38]. Finally, up to 90% of brain PrPSc in sCJD eludes WB analysis because it is destroyed by proteinase-K treatment, which is necessary to eliminate PrPSc. Consequently, the conformation or role of this major protease-sensitive (s) fraction of PrPSc in the pathogenesis of the disease is a subject of speculation [30,39,40].

Aiming to advance our understanding of the molecular pathogenesis of human prion diseases, we used a conformation-dependent immunoassay (CDI) [15,30,41] to determine the conformational range and strain-dependent molecular features of sCJD PrPSc in patients who were homozygous for codon 129 of the PRNP gene. Even relatively minute variations in a soluble protein structure can be determined by measuring conformational stability in a denaturant such as Gdn HCl [42]. Utilizing this concept, we designed a procedure in which PrPSc is first exposed to denaturant Gdn HCl and then exposed to europium-labeled mAb against the epitopes hidden in the native conformation [15]. As the concentration of Gdn HCl increases, PrPSc dissociates and unfolds from native β-sheet-structured aggregates; and more epitopes become available to antibody binding. These experiments involve insoluble oligomeric forms of PrPSc, and denaturation of this protein is irreversible in vitro; consequently the Gibbs free energy change (ΔG) of PrPSc cannot be calculated [43]. Therefore we chose instead to use the Gdn HCl value found at the half-maximal denaturation ([GdnHCl]1/2) as a measure of the relative conformational stability of PrPSc. The differences in stability reveal evidence of distinct conformations of PrPSc [15,42,43]. Because CDI is not dependent on protease treatment, it allowed us to address fundamental questions concerning the concentration and conformation of different isoforms of sCJD PrPSc, including protease-sensitive (s) and protease-resistant (r) PrPSc. We found a broad spectrum of structures that are likely responsible for the phenotypic heterogeneity of sCJD and we identified the structural characteristics of PrPSc that are linked to the duration of the disease.

Results

Diagnostic classification of sCJD patients homozygous for PRNP codon 129 and disease duration

From 340 patients with an unequivocally definite diagnosis of Type 1 or Type 2 sCJD and who were homozygous for codon 129 polymorphism in the PRNP gene, we selected samples from 46 patients. The descriptive statistics and Kaplan-Meier survival curves indicate that these cases are representative of the whole group collected at NPDPSC and are similar to those previously reported by us and others (Compare Figure 1 and Figure S1, Table 1) [21,38,44]. As expected, we did not observe statistically significant differences in sex ratio or age at onset of the disease [21,44]. Kaplan-Meier analyses of survival (Figure 1) demonstrated that patients with PrPSc Type 1 had a significantly shorter disease duration than patients with PrPSc Type 2 (P = 0.002) despite identical codon 129 MM polymorphism, age, and sex distribution (Table 1). Moreover, there is an apparent tendency toward longer survival of patients with Type 2 rPrPSc(129 V) than patients with Type 1 rPrPSc(129 M) (P = 0.017). The difference in survival between patients with Type 2 rPrPSc(129 V) and Type 2 rPrPSc(129 M) was also significant (P = 0.008) with shorter survival of those homozygous for valine (Figure 1).

To ensure that the brain homogenate analyzed by CDI contained only Type 1 or 2 rPrPSc, each brain homogenate underwent a second WB (Figure S2). The results confirmed the original diagnostic classification but we found two atypical patterns: Case #833 (Type 2 PrPSc(129 M) and Case #162 (Type 2 PrPSc(129 V) revealed, in addition to a band of unglycosylated rPrPSc with apparent molecular mass ~19 kDa, a second band with electrophoretic mobility corresponding to mass ~17 kDa. The observation of different glycoform patterns of PrPSc in different sCJD cases before protease K treatment and distinct resistance to proteolytic degradation of different glycoforms of PrPSc is interesting and deserves further investigation.

Measurement of PrPSc, sPrPSc, and rPrPSc in sCJD cortex by CDI

To measure the concentration of different forms of PrPSc in the frontal cortex, we used europium-labeled mAb 3F4 [45] for detection and 8H4 mAb (epitope residues 175–185) [46] to capture human PrPSc in a sandwich CDI format (Figure S4) [30,47]. The analytical sensitivity and specificity of the optimized CDI for detection of both protease-sensitive (s) and protease-resistant (r) conformers of PrPSc was previously reported by us and others in numerous publications [15,30,41,48–50] and has been...
shown to be as low as ~500 fg (20 attomoles) of PrPSc. This sensitivity of CDI is similar to the sensitivity of human prion bioassay in TgMHu2M/5378/Prnp0/0 mice [30].

First, we determined the concentration of disease-causing PrPSc in subpopulations of sporadic sCJD patients (Table 1 and Figure 2). We observed wide interindividual variations, and approximately sixfold more accumulated PrPSc in the frontal cortex of patients with Type 2 PrPSc(129 M) than those with Type 1 PrPSc(129 M) or Type 2 PrPSc(129 V). A large portion of PrPSc in all groups is protease-sensitive, constituting a pool of sPrPSc conformers (Table 1 and Figure 3a). The digestion with proteinase K (PK) was performed with 3 IU/ml (100 μg/ml) of 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 [15,30,38,40,47,51]. Additionally, the complete digestion of the PrPSc N-terminus with PK was monitored on WBs in all samples (Figure S2).

In patients with Type 2 PrPSc(129 M), significantly higher concentrations of total PrPSc and sPrPSc protein (Table 1) are associated with extended duration of disease. However, the concentration of sPrPSc varies greatly between individual patients, with numerous overlapping values between each classification group (Figure 3a). Thus, the concentration of sPrPSc is expressed as a percentage of total PrPSc, with no significant difference between groups appears, and the proportion of sPrPSc varies from 5% to 90% in individual patients (Figure 3b). We concluded from these observations that a major portion of pathogenic sCJD PrPSc is protease-sensitive and that the highest levels of sPrPSc are present in Type 2 PrPSc(129 M). The observed large interindividual differences in PK sensitivity likely indicate a broad range of PrPSc conformers within each PRNP genotype and WB pattern [15,39]. Since the proteolytic sensitivity of PrPSc is considered a reliable and constant marker of a distinct prion strain, the data support the conclusion that distinct prion structures are present within each classification group.

Monitoring the exposure of epitopes 108–112 and 175–185 in native sCJD PrPSc

The partial exposure of epitopes 108–112 and 175–185 in native pathogenic PrPSc reflects differences in the conformation of native PrPSc [15,52]. When we adopted this approach previously, we found considerable differences among eight laboratory prion strains passaged in Syrian hamsters [15]. The denatured state is a reference corresponding to the concentration of PrPSc; the ratio between the fluorescence signal of europium-labeled mAb 3F4 reacting with PrPSc in the native (N) or completely denatured (D) state represents a relative measure of the degree of exposure of these epitopes.

The highest D/N PrPSc ratio was found in patients with Type 2 PrPSc(129 M); and despite a large spread of values, the difference is statistically significant (P = 0.002) (Figure 4). PK treatment eliminated most of the exposed 108–112 and 175–185 epitopes in patients with Type 1 PrPSc(129 M) and in patients with Type 2 PrPSc(129 V), resulting in the increased D/N ratios (Figure 4). The opposite trend was observed in patients with Type 2 PrPSc(129 M). After PK treatment the PK-induced differences
Table 1. Demographics of sCJD patients and descriptive statistics of the data.

| Variable | Units | n | Min | Max | Mean | SEM | Sig |
|----------|-------|---|-----|-----|------|-----|-----|
| **Sex**  |       |   |     |     |      |     |     |
| M        |       | 10 | 5.0 | 9.2 | 7.59 | 2.2 | NS  |
| F        |       | 6  | 5.0 | 9.2 | 7.59 | 2.2 | NS  |
| **Age**  | years | 16 | 14  | 32 | 26.1 | 3.1 | NS  |
| **Disease Duration** | months | 16 | 11  | 20 | 13.6 | 2.2 | NS  |
| **PrP Sc(129 M)** | pmol/ml | 11 | 22  | 547 | 196 | 53 | 0.001 |
| **PrP Sc(129 V)** | pmol/ml | 11 | 23  | 547 | 196 | 53 | 0.001 |
| **PrP Sc** | % | 14 | 21  | 50 | 32.5 | 4.2 | NS  |
| **PrP Sc** | % | 14 | 21  | 50 | 32.5 | 4.2 | NS  |
| **PrP Sc** | % | 14 | 21  | 50 | 32.5 | 4.2 | NS  |
| **PrP Sc** | % | 14 | 21  | 50 | 32.5 | 4.2 | NS  |
| **PrP Sc** | % | 14 | 21  | 50 | 32.5 | 4.2 | NS  |
| **PrP Sc** | % | 14 | 21  | 50 | 32.5 | 4.2 | NS  |

The error of the method does not exceed 5% in monitoring pellet, regardless of protease sensitivity or prion strain [15,30,53–55]. The possible cluster of marginally significant (P = 0.040) and there is no statistically significant difference among other groups. The increased frequency of exposed epitopes in codon 129 MM samples with Type 2 rPrPSc after PK treatment is unexpected and may indicate one of three possibilities: that the ligand protecting the 3F4 epitope was removed by PK treatment; that epitope 108–112 was protected by the N-terminus of PrPSc; or that conformational transition resulted in more exposed 108–112 epitopes. 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.

Dissociation and unfolding of sCJD PrPSc, sPrPSc, and rPrPSc monitored by CDI

First, we asked whether the PTA precipitation has an impact on the stability of PrPSc. This step in the protocol was important for eliminating high concentrations of PrPSc and for concentrating PrPSc in brain samples with relatively low levels of PrPSc. Figure S5. The denaturation curves performed on 5% brain homogenate before PTA precipitation, on PTA pellet and on PTA pellet washed with an excess of H2O, were superimposable, an effect which indicated that PTA quantitatively concentrated all PrPSc conformers and did not influence the stability in CDI. This conclusion accords with numerous previously published data, including bioassays, which indicate that PTA does not precipitate PrPC and recovers specifically including bioassays, which indicate that PTA does not precipitate PrPC and recovers specifically [38].

Since the dissociation and unfolding of oligomeric PrPSc may be dependent on protein concentration [42], we first followed the process with CDI at different dilutions of PrPSc (Figure S5). The resulting overlapping dissociation/unfolding curves of PrPSc with variation in Gdn HCl1/2 values <3% indicate that in the 10–250 ng/ml concentration range, the dissociation/unfolding is independent of concentration and is highly reproducible. Furthermore, to ensure the same conditions in all dissociation/unfolding experiments, the PrPSc content in all samples was maintained at a constant 50 ng/ml concentration. As we observed previously with the western blot technique, the Gdn HCl1/2 values obtained with frontal, temporal, parietal, and occipital cortex, thalamus, and cerebellum in three typical sCJD cases were superimposable, indicating that the same conformers of PrPSc are present in different anatomical areas (data not shown) [38].

Next we examined the frontal cortex of individual sCJD patients homozygous for methionine or valine at codon 129 of the PRNP gene. Typical examples of dissociation/unfolding curves are shown in Figures 3a, 3b, and 3c. Comparing all sCJD cases, we found a broad range of Gdn HCl1/2 values ranging from 1.3 to 3.5 M (Figure 6a). Because of the wide spread of values, the difference between the cases with Type 1 and 2 PrPSc(129 M) is only marginally significant (P = 0.040) and there is no statistically significant difference among other groups. The possible cluster of Gdn HCl1/2 values at ~3.0 M is discernible in cases with Type 1 PrPSc(129 M) (Figure 6a). We concluded from these experiments that PrPSc proteins in different brains of sCJD patients display a vast range of unique conformations within each classification group.
The conformational impact of PK treatment

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 PrPSc with PK resulted in increased conformational stability in Type 1 rPrPSc(129 M) and Type 2 rPrPSc(129 V) but did not significantly reduce the range of values (Figure 6a). In contrast, PK treatment uniformly decreased Gdn HCl1/2 values in Type 2 rPrPSc(129 M) (Figure 6a). The marked drop in this group’s stability is statistically significant to a high degree (P, 0.001). Additionally, there is a discernible cluster of Type 2 PrPSc(129 M) cases at 2.6 M (Figure 6a). We interpret the data as providing evidence of a wide range of unique conformations in each subgroup. Proteolytic treatment selects the conformers having a more stable core in Type 1 rPrPSc(129 M) and Type 2 PrPSc(129 V). The opposite effect of PK, as well as decreased stability, was observed in samples with Type 2 PrPSc(129 M). These data suggest that PK treatment generates a unique set of conformers in Type 2 rPrPSc(129 M), characterized by increased exposure of 108–112 and 175–185 epitopes (Figure 5) and, upon PK treatment, decreased stability of the core rPrPSc(129 M).

To investigate the conformational stability of sPrPSc separately from rPrPSc, we subtracted the relative fractional change in stability of rPrPSc after PK treatment from the PrPSc values obtained before PK (Figures 5a, 5b, and 5c). The resulting 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. Overall stability of Type 1 sPrPSc is, as expected, lower than that of rPrPSc and we estimate, from these data alone, that sPrPSc conformers constitute 13–72% of the PrPSc (Figure 6a). A larger spread of positive values obtained with Type 2 sPrPSc(129 V) coincides with a generally larger spread of Gdn HCl1/2 values in this group. In contrast, the negative differential curves for Type 2 sPrPSc(129 M) indicate that sPrPSc is more stable than rPrPSc in this patient group (Figure 6b). Notably, the only positive value in this group came from a sample having an atypical 19 and 17 kDa doublet of unglycosylated rPrPSc on WBs (Figure 6b and Figure S2). Since the stability of sPrPSc and of rPrPSc reflect different initial conformation, the observed spread of values suggests a broad range of unique PrPSc conformers within each PRNP genotype and WB pattern [15,39,56].

To determine whether unifying trends exist, we examined which PrPSc characteristics have an impact on duration of the disease in individual patients in all groups using regression analysis. In contrast to analysis of variance (Anova) used to compare MM1, MM2, and VV2 groups (Table 1), the regression analysis is testing the relationship between a dependent variable (duration of the disease) and independent variables (e.g., sPrPSc levels) in individual patients. From concentrations of PrPSc, sPrPSc, and rPrPSc, only the levels of sPrPSc (Figure S7a) correlated significantly with longer duration of the disease. The overall dependency is driven mainly by the higher levels of sPrPSc in Type 2 sPrPSc(129 M) and longer duration of the disease in this subgroup (Table 1). Additionally, the measurement of absolute concentration of sPrPSc is clearly a better indicator of this relationship than the estimate of the relative fraction (percentage) of sPrPSc (Figure 3b). Despite a wide spread of values, this
observation corroborates the conclusion, drawn from previous experiments with eight laboratory strains of prion, that incubation time, and by extension duration of the disease, is linked to the higher levels of sPrPSc [15].

We then analyzed the conformational characteristics of PrPSc. The stability of rPrPSc clearly did not correlate with duration of the disease in individual cases (Figure 7a). In contrast, the change in the stability of PrPSc upon PK treatment (Figure S7b) or relative levels of sPrPSc conformers eliminated by PK (Figure 7b) expressed as a fraction of all conformers, both demonstrated better correlation with duration of the disease than did any other parameter in both Type 1 and Type 2 cases. In contrast to simple measurement of sPrPSc concentration, the stability assay performed before and after PK treatment cumulatively determines...
the shift in the stability of PrPSc, change in the slope of the denaturation curve (dissociation/unfolding rate), and relative levels of the sPrPSc conformers in the total PrPSc pool. This effect leads to the clear separation of Type 1 from Type 2 sPrPSc(129 M) cases (Figure 7b). We interpret these findings as evidence of the differential impact of protease treatment on different conformers, resulting in either increased or decreased stability of the remaining rPrPSc core (PrP 27–30). Taken together, higher levels of more stable sPrPSc conformers are associated with extended duration of the disease. Conversely, lower concentrations of unstable sPrPSc correlate with faster progression of the disease.

Discussion

The discovery of heritable polymorphic PK cleavage sites and glycosylation patterns in PrPSc have been used for the initial diagnostic classifications of sCJD cases. In concert with the codon 129 PRNP haplotype, the different rPrPSc types broadly correlate with distinct disease phenotypes [14,21,27–29,57]. The majority of sCJD patients are homozygous for methionine at codon 129 of the PRNP gene; they also accumulate Type 1 rPrPSc and present with so-called classic sCJD, characterized by rapidly progressive dementia, early myoclonus, visual disturbances including cortical blindness, disease duration of approximately 4 months, and fine punctate (synaptic) deposits of PrPSc [21,30]. In contrast, patients with the second most frequent phenotype are homozygous for valine at codon 129 of the PRNP gene, accumulate Type 2 PrPSc and manifest a different disease course, with early ataxia, predominant extra-pyramidal symptoms, relatively late-onset dementia in the extended course of the disease, and large plaque-like deposits of PrPSc [21].

In the increasing number of subsequent sCJD cases which were examined with more sensitive and specific techniques, investigators began to recognize the extensive variability of the sCJD phenotypes, as well as the extreme complexity of brain immunohistochemistry and western blot patterns of PrPSc [25,32,37,38,57–59]. Although the western blot systems provided early evidence that molecular characteristics of PrPSc are transmissible, evidence regarding the original conformation of PrPSc remains indirect and limited to the most protease-resistant fractions. Because variable fractions of PrPSc are protease-sensitive, we decided to determine the conformational characteristics directly, by using CDI. This method allowed us to compare the conformational features of human PrPSc independently of proteolytic treatment and in addition provided quantitative data on levels of PrPSc, sPrPSc, and rPrPSc [15,30]. The CDI techniques represent a major improvement over previously used semi-quantitative WB-based methods, the finding that has been independently confirmed by another group [60,61]. The dissociation and unfolding of PrPSc in a presence of increasing concentration of Gdn HCl can be described as follows: [PrPSc]_n→[sPrPSc]_n→iPrP→uPrP, where [PrPSc]_n are native aggregates of PrPSc, [sPrPSc]_n are soluble protease-sensitive oligomers of PrPSc, iPrP is an intermediate, and uPrP is completely unfolded (denatured) PrP [7,43,62]. The CDI monitors the global transition from native aggregates to fully denatured monomers of PrPSc. In contrast, the WB based techniques monitor either the partial solubilization of PrPSc [63] or conversion of rPrPSc to protease-sensitive conformers [16] after exposure to denaturant. As a result, the stability data on soluble protease sensitive oligomers and intermediates of PrPs cannot be obtained with WB techniques and lead to the markedly underestimated values [60].
Levels and role of PrP<sup>Sc</sup> isoforms in the pathogenesis of sCJD

The sixfold difference in concentrations of PrP<sup>Sc</sup> between Type 1 and Type 2 PrP<sup>Sc</sup>(129 M) ([Figure 2](#)) revealed in the frontal cortex by means of CDI was surprising, even though some variability was to be expected due to differences in the predominantly affected areas in distinct sCJD phenotypes [30]. The average levels of PrP<sup>Sc</sup> are up to 100-fold lower than those in standard laboratory prion models such as Syrian hamsters infected with Sc237 prions [15]; and together with the up to 100-fold variability within each phenotypic group, these lower levels of...
PrPSc may partially explain why some sCJD cases are difficult to transmit, and why lower endpoint titers are obtained with human prions in transgenic mice expressing human PrPC [14,26,30,64]. As we observed previously, up to 90% of the pathogenic prion protein was protease-sensitive [30]. In this study, we found the highest concentrations in Type 2 PrPSc(129 M). The broad range of absolute and relative levels of rPrPSc and sPrPSc offers evidence of a broad spectrum of PrPSc molecules differing in protease sensitivity in each group with an identical polymorphism at codon 129 of the PRNP gene and an identical WB pattern (Figure 3). Moreover, these findings signal the existence of a variety of sCJD PrPSc conformers; and since protease sensitivity is one of the

Figure 6. Summary data on conformational stability of PrPSc, rPrPSc, and change in stability induced by PK in 46 sCJD cases. The (a) conformational stability of PrPSc before (blue) and after (red) PK digestion and (b) fractional change in stability of PrPSc induced by PK conformers in individual sCJD samples (circles) Type 1 PrPSc(129 M), (squares) Type 2 PrPSc(129 M), and (diamonds) Type 2 PrPSc(129 V). The stability was determined by CDI and expressed as Gdn HCl1/2 or stability change \( \Delta F \) induced by PK. Each symbol represents an individual patient measured in triplicate and the mean level in each group is indicated by the horizontal line.

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characteristics of prion strains, they also suggest that distinct sCJD prion strains exist [15,30,31,58,62,65].

**Structural heterogeneity and origin of sCJD PrP<sub>Sc</sub>**

The CJD cases studied in this paper represent 75–90% of all clinical and pathologic diagnostic categories of sCJD [21]. In order to allow unequivocal interpretation of the CDI data, we had to exclude sCJD patients heterozygous for codon 129 polymorphism in the PRNP gene, even though they represent 15–20% of sCJD cases. The CDI cannot differentiate PrP<sup>Sc</sup> with codon 129 M from V in a mixture which is present in sCJD heterozygots, and therefore we were unable to differentiate the conformational impact of codon 129 polymorphism. We also excluded the VV1 type of sCJD because of its rarity. This rare form of sCJD constitutes ~1% of all sCJD cases and we did not collect enough cases to allow statistical comparison with the other groups [21].

The heterogeneity of PrP<sup>Sc</sup> conformations found with CDI within sCJD patients homozygous for codon 129 polymorphism of the PRNP gene is remarkable (Table 1 and Figure 6), with a range corresponding to that of stabilities found in more than ~30 distinct strains of de-novo and natural laboratory rodent prions studied up to now [15,16,66]. The high sensitivity and reproducibility of CDI, together with broad inter-individual variability detected with techniques based on three different principles—PK sensitivity, epitope exposure, and conformational stability—all indicate that the intragroup variations did not originate in the CDI technique but rather reflect differences in the structure of PrP<sup>Sc</sup> in different patients. The intriguing effect of

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**Figure 7. Duration of sCJD correlate with conformational stability of sPrP<sup>Sc</sup>.** The relationship between duration of the disease and conformational stability of (a) rPrP<sup>Sc</sup> and (b) fraction of sPrP<sup>Sc</sup> conformers in 46 sCJD patients was analyzed by the regression analysis. doi:10.1371/journal.ppat.1002242.g007
PK treatment on the stability of Type 2 PrPSc(129 M) suggests that the protease-resistant core of Type 2 was profoundly destabilized. Since sCJD cases with Type 2 PrPSc(129 M) have remarkably extended disease durations, the molecular mechanism underlying this effect calls for detailed investigation.

Several theories have been proposed to explain the origin of sCJD. One argues for spontaneous somatic mutations in PRNP; another, for rare stochastic conformational changes in PrPSc [26,67]. Yet a third hypothesis holds that low levels of PrPSc are normally present and cleared, but rise to pathogenic levels when the clearance mechanism fails [40]. Cumulatively, our findings indicate that sCJD PrPSc exhibit extensive conformational heterogeneity. Whether this heterogeneity originates in a stochastic misfolding process that generates many distinct self-replicating conformations [26,67] or in a complex process of evolutionary selection during development of the disease [17] remains to be established.

Protease-sensitive conformers of PrPSc

We discovered this fraction of PrPSc while developing a conformation-dependent immunoassay (CDI), which does not require proteolytic degradation of ubiquitous PrPSc [15]. Although the original definition of sPrPSc was only operational, considerable additional data demonstrate that (1) sPrPSc replicates in vivo and in vitro as an invariant and major fraction of PrPSc [4]; (2) sPrPSc separates from rPrPSc in high speed centrifugation; and (3) the proteolytic sensitivity of PrPSc can reliably differentiate various prion strains [15,30,31,58,62,65]. Accumulation of sPrPSc precedes protease-resistant product (rPrPSc) in prion infection [40,68]; and up to 90% of PrPSc accumulating in CJD brains consists of sPrPSc [30]. Thus, the detection by CDI of sPrPSc as a disease-specific marker is widely regarded as a more reliable basis for diagnosing prion diseases. This improved detection led to the discovery of a new human prion disorder, variable protease-sensitive prionopathy (VPSPr) [15,30,39,69,70]. It is noteworthy that protease-sensitive synthetic prions generated in vitro during polymerization of recombinant mouse PrP into amyloid fibers produced upon inoculation into wild mice prions composed exclusively of sPrPSc [66].

In laboratory rodent prion models, we found that levels of sPrPSc varied with the incubation time of the disease [13] but the molecular mechanism of this link was unknown [15,30,40]. Subsequent experiments with yeast prions indicated that replication rate may be an inverse function of the stability of misfolded protein [71]. The hypothesis based on these experiments posits that the least stable prions replicate faster by exposing more available sites for growth of the aggregates. Additionally, experiments with laboratory and synthetic prions in mouse suggested that the yeast prion principle may apply to mammalian prions as well. However, these experiments were based entirely on the correlation of the shorter incubation time of mouse inoculated with PrPSc that on WBs converted to protease-sensitive isoforms at a lower denaturant concentration, whereas the replication rates of mammalian prions were never determined [72].

In this paper we determined the conformational features and stability of human sPrPSc in sCJD. The data indicate that the levels as well as stability are linked to the progression rate of the disease. Despite the inevitable influence of variable genetic background and the potential difficulties in evaluating initial symptoms, the disease progression rate and incubation time jointly represent an important parameter, which is influenced by replication rate, propagation, and clearance of prions from the brain [2,40]. The correlations among the levels of sPrPSc, the stability of sPrPSc, and the duration of the disease found in this study all indicate that sPrPSc conformers play an important role in the pathogenesis. When sPrPSc is less stable than rPrPSc, the difference in stability correlates with less accumulated sPrPSc and shorter duration of the disease. Conversely, when sPrP conformers are more stable than rPrPSc, we observe the opposite effect—more accumulated sPrPSc and extended disease duration. It remains to be determined if these effects represent an outcome of different replication rates and clearance, or whether they stem from as yet unknown aspects of the pathogenesis of sCJD.

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 patient or legal guardian and the material used had appropriate ethical approval for use in this project. All patient’s data and samples were coded and handled according to NIH guidelines to protect patients’ identities.

Patients and clinical evaluations

We selected 46 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 [73–75] and clearly determined and dated initial symptoms upon neurological examination to ascertain the disease duration; (2) methionine or valine 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, OH; (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 chart 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 [73–75]. 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 [29,30,76]. 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(129 M, n = 16); (2) Type 2 PrPSc(129 M, n = 16); or (3) Type 2 PrPSc(129 V, n = 14). Patients lacked pathogenic mutations in the PRNP and had no history of familial diseases or known exposure to prion agents. These cases underwent additional detailed WB analyses of the PrPSc so that we could ascertain the accuracy of their original classification and confirm that the same brain homogenate analyzed by CDI contained pure Type 1 PrPSc(129 M), Type 2 PrPSc(129 M), and Type 2 PrPSc(129 V).

Coronal sections of human brain tissues were obtained at autopsy and stored at 80°C. Three 200–350 mg cuts of frontal (superior and more posterior middle gyrus) cortex were taken from each brain and used for molecular analyses. The other symmetric
Brain homogenates and precipitation of prions with PTA

Slices of tissues weighing 200–350 mg were first homogenized to a final 15% (w/v) concentration in calcium- and magnesium-free PBS, pH 7.4, by 3 75 s cycles with Mini-beadbeater 16 Cell Disrupter (BioSpec, Bartlesville, OK). The homogenates were then diluted to a final 5% (w/v) in 1% (v/v) sarkosyl in PBS, pH 7.4 and rehomogenized. After clarification at 500× g for 5 min., one aliquot of the supernatant was treated with protease inhibitors (0.5 mM PMSF and aprotinin and leupeptin at 5 ug/ml, respectively). The second aliquot was treated with 30 µg/ml of proteinase K (Amresco, Solon, OH) for 1 h at 37°C shaking 600 rpm on Eppendorf Thermomixer (Eppendorf, Hauppauge, NY) and PK was blocked with PMSF and aprotinin-leupeptin cocktail. Both aliquots were precipitated with final 0.32% (v/v) NaPTA after 1 h incubation at 37°C as described [15]. The samples were spun 30 min at 14,000× g in Allegra X-22R tabletop centrifuge (Beckman Coulter, Brea, CA) and the pellets were resuspended in 250 ul of deionized water containing protease inhibitors (0.05 mM PMSF, aprotinin and leupeptin at 1 ug/ml each, respectively, and stored for analysis at −80°C.

Western blots

Both PK-treated and untreated samples were dissolved 9-fold in 1 x Laemmli Buffer (Bio-Rad, Hercules, CA) 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-HCl, SDS-PAGE gels (Bio-Rad) mounted in Bio-Rad Western Blot apparatus. After electro-transfer to Immobilon-P Transfer Membranes (Millipore, Bedford, MA), the 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/ICP (Sigma, St. Louis, MO). The PVDF membranes were developed with 0.05 µg/ml of biotinylated mAb 3F4 (Covance, Princeton, NJ), followed by 0.0175 ug/ml Streptavidin-Peroxidase conjugate (Fisher Scientific, Pittsburgh, PA) or with ascitic fluid containing mAb 3F4 (kindly supplied by Richard Kascsak) diluted 1:20,000 followed by Peroxidase-labeled sheep anti-mouse IgG Ab (Amersham, Piscataway, NJ) 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-dependent immunoassay (CDI)

The CDI for human PrP was performed as described previously [30,47], with several modifications. First, we used white Lumitrac 600 High Binding Plates (E&K Scientific, Santa Clara, CA) coated with mAb 8H4 (epitope 175–185) [16] 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, MA). Finally, the captured PrP was detected by a europium-conjugated [15] anti-PrP mAb 3F4 (epitope 108–112) [45] and the time-resolved fluorescence (TRF) signals were measured by the multi-mode microplate reader PHERAstar Plus (BMG LabTech, Durham, NC). The recHuPrP90–231,129 M and PrP(23–231) were used as a calibrant in the CDI assay from Witold Surewicz, and preparation and purification have been described previously [77]. The initial concentration of recombinant human PrP(23–231) and PrP(90–231) was calculated from absorbance at 280 nm and molar extinction coefficient 56650 M⁻¹ cm⁻¹ and 21640 M⁻¹ cm⁻¹, respectively. The purified recombinant proteins were dissolved in 1 M GdnHCl and 50% Stabilcoat (SurModics, Eden Prairie, MN), and stored at −80°C. The concentration of PrP was calculated from the CDI signal of denatured samples using calibration curve prepared with either recPrP(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(23–231,129 M) compared to PrP(90–231,129 M) (Figure S3).

Monitoring dissociation and unfolding of PrPSc by CDI

The denaturation of human PrPSc was performed as described previously [15], with several modifications. Frozen aliquots of PrPSc were thawed, sonicated 5×5 s at 60% power with Sonicator 4000 (Quonica, Newton, CT), 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, MO) 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, MA) containing diminishing concentrations of 8 M GdnHCl, so that the final concentration in all samples was 0.411 M. Each individual aliquot was immediately loaded in triplicate to dry white Lumitrac 600, High Binding Plates (E&K Scientific, Santa Clara, CA), coated with mAb 8H4, and developed in accordance with CDI protocol using europium-labeled mAb 3F4 for detection [15,30,41,78].

The raw TRF signal was converted into the apparent fractional change of unfolding (Fapp) as follows: F = (TRFobs−TRFk)/ (TRFobs−TRFk) where TRFobs is the observed TRF value, and TRFk and TRF0 are the TRF values for native and unfolded forms, respectively, at the given Gdn HCl concentration [7]. To determine the concentration of Gdn HCl where 50% of PrPSc is unfolded ([Gdn HCl]1/2), the data were fitted by least square method with a sigmoidal transition model (Equation 1):
disease; electrophoretic Type of PrP 27–30; and the concentration and stability of PrPSc in Gdn HCl before and after PK treatment [15]. Cumulative survival curves were constructed by the Kaplan-Meier method, both overall and by stratifying for each of the above variables. For each type of PrPSc and PRNP gene polymorphism, we report descriptive statistics and the overall survival times stratified for each variable. In the comparison of different patient groups, P values were calculated using Anova. Comparisons of survival curves among groups were carried out by the log rank (Mantel-Cox) and generalized Wilcoxon test. To evaluate the dependency of disease duration upon the concentration and stability of PrPSc in individual CJD cases, the data were analyzed by non-linear regression using the logistic function or the nonlinear models with the best fit. To obtain significance and to compare the relative importance of each characteristic of PrPSc, we used ANOVA and F statistics with regression mean square (MSR) divided by the residual mean square (MSE). All the statistical analyses were performed using SPSS 17 software (SPSS Inc., Chicago, IL).

Supporting Information

Figure S1 Kaplan-Meier cumulative survival analysis of 340 sCJD cases homozygous for either methionine (n = 286) or valine (n = 52) in codon 129 of PRNP gene from which were selected the 46 cases described in this paper. The sCJD cases carrying pure type 1 PrPSc(129 M) (n = 266) have significantly shorter disease duration than those with type 2 PrPSc(129 M) (n = 22, P < 0.001). The intermediate duration of the disease observed in sCJD cases with type 2 PrPSc(129 V) (n = 52) is significant compared with type 1 PrPSc(129 M) (P < 0.001) or type 2 PrPSc(129 V) (P < 0.001).

Figure S2 Typical WB analysis of PrPSc and rPrPSc in sCJD cases. PrPSc from 5% brain homogenate in PBS, pH 7.4, containing 1% Sarcosyl was precipitated with PTA either before (left lanes) or after (right lanes) digestion with 50 μg/ml of PK at 37°C for 1 h. Note the 19 and 17 kD doublets of unglycosylated bands of PrPSc in MM2 Case #7-927 and VV2 Case #8-848. The rPrPSc bands in Case VV2 9-434 became visible only after prolonged exposure (data not shown). Internal controls of type 1 (T1) or type 2 (T2) rPrPSc(129 M) were incorporated in each WB.

Figure S3 Calibration of CDI with (squares) full length (PrP23–231, 129 M) or (circles) truncated (PrP90–231, 129 M) prion protein. The truncated (PrP90–231, 129 M) prion protein corresponds to the human brain PrP 27–30 after proteinase K treatment. Time-resolved fluorescence (TRF) is reported in counts per minute (cpm) from triplicate measurement ± SEM. The initial concentrations of recombinant human PrP(23–231) and PreP(90–231) were calculated from absorbance at 280 nm and molar extinction coefficient 56650 M⁻¹ cm⁻¹ and 21640 M⁻¹ cm⁻¹, respectively.

Figure S4 The (a) raw time-resolved fluorescence (TRF) data and (b) end-point sensitivity in detection of sCJD PrPSc with CDI before and after proteinase K treatment in different cases of sCJD and a case of other neurological disorder (OND). To obtain values for total PrPSc, CDI was performed in an aliquot of brain homogenate that was precipitated in the presence of a protease inhibitor cocktail with PTA. To obtain CDI readings for rPrPSc, samples were treated with PK at concentration equivalent to 5 IU/ml (100 μg/ml) of 10% brain homogenate for one hour at 37°C and precipitated with PTA after blocking PK with the protease inhibitor cocktail. The 8H4 mAb was used (Zanusso, 1998 #4838) for capture and Eu-labeled 3F4 mAb for detection under native (N) and denatured (D) conditions (Safar, 2005 #6826; Safar, 2002 #5989; Safar, 1998 #4776). The D – N values of time-resolved fluorescence (TRF) measured in counts per minute (cpm) are directly proportional to the concentration of PrPSc [Safar, 2005 #6826; Safar, 2002 #5989; Safar, 1998 #4776]. Data points and bars represent average ± standard deviation (SD) obtained from three or four independent measurements.

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Author Contributions

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

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Supporting Information

Figure S5 The dissociation and unfolding of PrPSc(129 M) monitored by CDI in 5% brain homogenate (circles), in PTA pellet (squares), and washed PTA pellet (triangles). The brain homogenate and PTA precipitation was performed as described in the Method section. For wash, the PTA pellet was resuspended in 1 ml of H2O containing protease inhibitors, spun at 14,000 G for 30 min, and then processed as described for the other samples. To obtain accurate midpoint of the curves from raw TRF data requires the least square fit of the sigmoidal transition model (Equation 1).

Figure S6 The dissociation and unfolding of rPrPSc monitored by CDI at different concentrations. The (a) row data with TRF or (b) values of apparent fractional change (Fapp) at each concentration of Gdn HCl in each dilution are mean ± SEM obtained from triplicate CDI measurements. Note the logarithmic scale in the plot that was necessary due to the 1000-fold range of TRF values but made the manual estimate of the Gdn HCl1/2 difficult. To obtain accurate midpoint of the curves from raw TRF data, we used the least square fit of the sigmoidal transition model (Equation 1) or Fapp transformation. Both methods gave indentical results.

Figure S7 The relationship between duration of the disease and (a) concentration of sPrPSc or (b) change in the stability of PrPSc after PK digestion in all sCJD patients (n = 46). The regression analysis was performed by using data from (a) Figure 3 and (b) Figure 6.
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