Chapter 12
Molecular Mechanisms Encoding Quantitative and Qualitative Traits of Prion Strains

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Abstract Yeast, fungal, and mammalian prions determine heritable as well as infectious traits. In mammals, prions cause a group of fatal and rapidly progressive neurodegenerative diseases, originally described as transmissible spongiform encephalopathies (TSEs). Variations in prions, which cause different disease phenotypes, are referred to as strains. Mammalian prion strains are differentiated by qualitative characteristics such as clinical symptoms, brain pathology, targeted brain anatomical areas and cells, or Western blot patterns of glycosylated or deglycosylated pathogenic prion protein (PrPSc). Quantitative prion traits are determined by incubation time, prion dose response, proteolytic sensitivity, and conformational stability of PrPSc. The high degree of fidelity with which prion strains replicate requires a precise molecular mechanism that can account for all these characteristics. Remarkable progress in the past decade produced many lines of evidence arguing that prion traits are encoded in the self-replicating conformation of PrPSc that is unique for each strain. Thus, prions behave like proteinaceous genes. The determination of the full spectrum of human and animal prion strains and the conformational features in the pathogenic human prion protein that govern replication of prion strains is essential for the development of diagnostic as well as therapeutic strategies.

Keywords Prion strains • Conformation of prion protein • Protein misfolding cyclic amplification (PMCA) • Conformation-dependent immunoassay (CDI) • Neurodegeneration

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W.-Q. Zou and P. Gambetti (eds.), *Prions and Diseases: Volume 1, Physiology and Pathophysiology*, DOI 10.1007/978-1-4614-5305-5_12,
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Abbreviations

ALS	Amyotrophic lateral sclerosis
CDI	Conformation-dependent immunoassay
CHO	N-linked complex glycosylation chains
CJD	Creutzfeldt–Jakob disease
CPA	Cell panel assay
ER	Endoplasmic reticulum
FFI	Fatal familial insomnia
GSS	Gerstmann–Sträussler–Scheinker syndrome
PMCA	Protein misfolding cyclic amplification
PRNP	Prion protein gene
PrP	Prion protein
PrP^{C}	Normal or cellular prion protein
PrP^{Sc}	Pathogenic prion protein
$rPrP^{Sc}$	Protease-resistant conformers of pathogenic prion protein (PrP 27-30)
sCJD	Sporadic Creutzfeldt–Jakob disease
SFI	Sporadic fatal insomnia
$sPrP^{Sc}$	Protease-sensitive conformers of pathogenic prion protein
SSCA	Standard scrapie cell assay
TSE	Transmissible spongiform encephalopathy
VPSPr	Variable protease-sensitive prionopathy
WB	Western blot

12.1 Prion Diversity

Unique characteristics of mammalian prion isolates, which cause distinctive disease phenotypes, are referred to as strains. Prion strains were initially isolated based on distinctive clinical symptoms in goats with scrapie (Pattison and Millson 1961). Subsequently, strains were isolated in rodents based on divergent incubation times and neuropathologic profiles (Fraser and Dickinson 1973; Dickinson and Fraser 1977). New strains have been produced upon passage from one species to another (Kimberlin et al. 1987), from nontransgenic (Tg) mice to mice expressing a foreign or artificial PrP transgene (Scott et al. 1997), or most recently in vitro from recombinant prion protein (Legname et al. 2006; Wang et al. 2010).

For several decades, the existence of several prion strains was offered as an argument for the existence of a scrapie-specific nucleic acid (Bruce and Dickinson 1987; Dickinson and Outram 1988). However, despite numerous attempts to find such a nucleic acid using several approaches and despite mounting evidence against the existence of a strain-coding polynucleotide (Meyer et al. 1991; Kellings et al. 1992, 1994; Safar et al. 2005a), an explanation for prion strains remained a conundrum and a major challenge to basic principles of molecular biology (Safar et al. 2005a; Prusiner 1998a; Weissmann 2004). Moreover, the discovery that different

strains of prions can be propagated indefinitely with high fidelity in inbred mouse lines expressing only a single PrP sequence and the finding that prion strains were selective with regard to the cells in which they can replicate raised fundamental questions (a) How many mammalian prion strains exist? (b) How can cells distinguish different prion strains, as reflected in the cells' ability to propagate them? (c) How are strain-specific characteristics encoded if the prion is composed solely of PrP with the same sequence?

12.2 Distinct Phenotypes of Prion Strains in Bioassay

An important milestone in the history of research on prion strains was the experimental transmission of scrapie from sheep to mice ~18 months after intracerebral inoculation of brain extracts (Chandler 1961). On second passage, the incubation periods shortened to 4–5 months and remained constant on subsequent passages. The demonstration that scrapie could be transmitted to a small laboratory rodent made possible many new experimental studies that were previously impracticable in sheep or goats and helped to identify and characterize the first prion isolates by distinct clinical symptoms, incubation time, and brain pathology (Fraser and Dickinson 1973; Dickinson et al. 1972). A second milestone occurred with the development of an incubation time bioassay in Syrian hamsters, which reduced the time required to measure prions in samples with high titers by a factor of nearly 6; only 70 days were required instead of the 360 days previously needed. Equally important, four animals could be used instead of the 60 mice that were required for endpoint titrations, and this made possible a large number of parallel experiments (Prusiner et al. 1982, 1999a). However, there were disadvantages to using hamsters instead of mice: (1) the number of inbred hamster strains was small, (2) they we susceptible to only some prion strains, and (3) there were no procedures for transfer and ablation of genes in the hamster. Thus, the third milestone became the production of transgenic (Tg) mice overexpressing prion protein homologous to the original prion host, for example, mouse (Mo), Syrian hamster, or human (Hu) PrP. In contrast to nontransgenic hosts, Tg mouse models of prion diseases produced the original species of prions, and overexpression of the PRNP gene led to significantly shorter incubation times (Carlson et al. 1994a; Scott et al. 1989). Most importantly, the transmission experiments established stable laboratory strains of prions with defined biological characteristics that became standard experimental tools in prion research (Prusiner et al. 1999a, 2004a, b; Scott et al. 2004).

Because of the wealth of data accumulated in animal experiments, the parameters distinguishing distinct mammalian prion isolates fell into qualitative or quantitative categories:

1. Qualitative traits:
   (a) Clinical symptoms of the host (Pattison and Millson 1961)
   (b) Anatomical distribution and characteristics of brain lesions (Fraser and Dickinson 1973; Dickinson and Fraser 1977)

(c) Anatomical distribution of pathogenic PrP^{Sc} in the brain (Gambetti et al. 2003; Taraboulos et al. 1992)
(d) Mass of unglycosylated or deglycosylated $rPrP^{Sc}$ on Western blots (WBs) (Parchi et al. 1996; Bessen and Marsh 1994; Telling et al. 1996)
(e) Glycoform pattern of $rPrP^{Sc}$ on WBs (Collinge et al. 1996)
(f) Conformational characteristics of PrP^{Sc} in conformation-dependent immunoassay (CDI) (Safar et al. 1998)

2. Quantitative traits:
(a) Incubation time (Pattison and Millson 1961)
(b) Dose–response curve in endpoint titration (Kimberlin and Walker 1978)
(c) Susceptibility of pathogenic PrP^{Sc} to proteases (Safar et al. 1998)
(d) Conformational stability of PrP^{Sc} (Safar et al. 1998, 2011; Peretz et al. 2001)

12.3 Prion Species

A prion species is defined by the amino acid sequence of the donor's (host's) PrP. Transmission of prions between different animal species frequently results in low transmission rates and long incubation times, which shorten upon repeated transmission to the recipient species (Scott et al. 2004; Safar et al. 2011; Bruce and Dickinson 1979). This so-called species barrier is attributed to differences in the PrP sequences between prion donor and new host that hinder the response of host PrP^{C} to the incoming $rPrP^{Sc}$ seed (Scott et al. 2004; Collinge and Clarke 2007). A "species barrier" may also exist within the same animal species; for example, there are two distinct polymorphic PrP alleles in different mouse lines, the Prnpa (108L, 189T) and the Prnpb allele (108F, 189V), and transfer of prions between mice with divergent PrP alleles is subject to a barrier similar to that observed in the transfer between different animal species (Prusiner et al. 2004a; Carlson et al. 1994b; Tremblay et al. 2004).

In the case of interspecies prion transfer to mice, the barrier may be overcome by replacing the murine PrP genes with their counterpart from the donor, for example, Syrian hamster (Prusiner et al. 1990), cattle (Scott et al. 1999), human (Telling et al. 1994), or cervids (Browning et al. 2004). Importantly, in PrP-deficient ($Prnp^{0/0}$) mice, neither prion disease nor prion replication has been found (Büeler et al. 1993). But replacement of the murine PrP gene with its homologs from another species does not recreate the physiology of the donor species, and genes other than PrP may play a role in susceptibility to prions, thereby resulting in different incubation times (Tamguney et al. 2008; Stephenson et al. 2000; Prusiner et al. 1999b). From these experiments and those in vitro, several authors have proposed an auxiliary role for an as yet hypothetical host-derived cofactor in prion replication, which could be a polynucleotide, glycosaminoglycan, lipid, or chaperone facilitating conversion (Kaneko et al. 1997; Kim et al. 2010; Deleault et al. 2010, 2012; Piro and Supattapone 2011; Geoghegan et al. 2007).

Cumulatively, the expression of foreign, mutant, or chimeric PrP transgenes in mice has created a wealth of knowledge about prions that was previously unattainable. Most importantly, this knowledge helped to separate the phenomena generated by "species barrier" from true strain characteristics encoded in the prion itself (Scott et al. 2004, 2005; Collinge and Clarke 2007). It has also helped to define the central domain (residues 96–167) in the PrP amino acid sequence determining "species barrier" (Scott et al. 2004), demonstrated an inverse relationship between the level of PrP^{C} expression and the incubation time (Scott et al. 1989), and allowed differentiation of the natural prion isolates from de novo prions generated with mutant and recombinant PrP (Legname et al. 2006; Wang et al. 2010; Tremblay et al. 2004; Safar et al. 2000).

12.4 Cell Tropism of Prion Strains

A few traits, such as clinical symptoms, pathology, and CNS distribution of pathogenic PrP^{Sc}, probably indicate distinct susceptibility of different cells to prions (Mahal et al. 2007). Different prion strains are evident in different locations of lesions and PrP^{Sc} deposition in the brain and may exhibit different tropism for cell lines (Mahal et al. 2007). Because the uptake of PrP^{Sc} by cultured cells appears to be a nonspecific process, the distinct susceptibility of various cells to different prion strains probably reflects the capacity of the cell to replicate prions at a rate exceeding natural clearance (Bergstrom et al. 2006; Mishra et al. 2004).

Some authors studying Western blot patterns of PrP 27-30 proposed that the observed differences in glycosylation specify prion strains (Collinge et al. 1996). However, this proposal is difficult to reconcile with the addition of high mannose oligosaccharides to Asn-linked consensus sites on PrP in the ER and subsequent remodeling of the sugar chains in the Golgi (Endo et al. 1989). Modification of the complex CHOs attached to PrP^{C} is clearly completed prior to the PrP^{C} trafficking to the cell surface (Borchelt et al. 1990; Caughey and Raymond 1991), which indicates that the Asn-linked CHOs of PrP^{Sc} do not instruct the addition of such complex-type sugars to PrP^{C}. Mutagenesis of the complex-type sugar attachment sites seemed to increase PrP^{Sc} formation in cultured cells (Taraboulos et al. 1990) but resulted in prolonged incubation times in Tg mice and differences in the patterns of PrP^{C} distribution and PrP^{Sc} deposition in mice expressing mutant PrPs (DeArmond et al. 1997; Tuzi et al. 2008). Finally, the idea that strain recognition is mediated by the nature of the glycans carried by PrP^{Sc} is not supported by the finding that two distinct prion strains could be propagated by PMCA using unglycosylated PrPC (Piro et al. 2009). Cumulatively these studies indicate that Asn-linked glycosylation might alter the stability and susceptibility of PrP^{C} to conversion, thereby resulting in distinctive patterns of PrP^{Sc} deposition and glycosylation on WBs.

An important contribution to the understanding of cellular phenomena related to prion strains came from the cell panel assay (CPA) developed by Charles Weissmann and colleagues. Conventionally the distinction between mouse-adapted prion strains

requires determination of incubation times in at least two mouse lines extending over 6–10 months. The CPA, which can distinguish between various murine prion strains in less than 2 weeks (Mahal et al. 2007), is based on the standard scrapie cell assay (SSCA), a method for the rapid and sensitive quantification of prions in vitro. The CPA carried out on a set of four cell lines, PK1, R33, CAD5, and LD9, showed different responses to various prions (Mahal et al. 2007; Karapetyan et al. 2009) and allowed reliable distinction of RML, 22L, 301C, and Me7 mouse prion strains. Additionally, when transferred from brain to cultured cells, "cell-adapted" prions outcompeted their "brain-adapted" counterparts, but the opposite occurred when prions were returned from cells to brain. Thus, the authors concluded that prions, although lacking a nucleic acid genome, are subject to mutation and selective amplification (Li et al. 2010).

However, the mechanisms underlying specificity for brain areas and for cultured cell lines in vitro are likely to be somewhat different. Persistent infection requires that the rate of PrP^{Sc} synthesis be at least equal to the rate of PrP^{Sc} depletion (Weissmann 2004). In cell culture, depletion of PrP^{Sc} is caused by degradation, secretion, and cell division, whereas in brain, where PrP^{Sc} accumulates predominantly in neurons, depletion does not occur by cell division. Thus, slowing cell division of cultured cells not only increases the accumulation of PrP^{Sc} but may also allow cells to become chronically infected by strains to which they are resistant under normal growth conditions (Ghaemmaghami et al. 2007). The fact that many drugs that "cure" chronically infected cell lines are largely ineffective in abrogating prion disease in vivo reflects at least in part the fact that in the brain PrP^{Sc} depletion does not occur by cell division (Ghaemmaghami et al. 2007; Collinge et al. 2009; Trevitt and Collinge 2006).

12.5 Conformational Mechanism of Prion Strain Propagation

Most researchers now accept the model according to which the infectious pathogen responsible for TSEs is pathogenic PrP^{Sc} (Prusiner 1982). This protein is a misfolded, β-sheet-rich isoform of the normal cellular prion protein, PrP^{C}, which is predominantly α-helical (Collinge and Clarke 2007; Prusiner 1998b, 2004; Caughey et al. 2009; Cobb and Surewicz 2009; Morales et al. 2007). The discovery that proteins may be infectious represents a new paradigm of molecular biology and medicine. Although originally deemed heretical, this protein-only model is now supported by a wealth of biochemical, genetic, and animal studies (Collinge and Clarke 2007; Prusiner 1998b, 2004; Caughey et al. 2009; Cobb and Surewicz 2009; Morales et al. 2007), including recent success in generating infectious prions in vitro (Wang et al. 2010; Kim et al. 2010; Legname et al. 2004; Castilla et al. 2005; Barria et al. 2009; Deleault et al. 2007; Geoghegan et al. 2009). The PrP^{Sc} conformer is believed to self-replicate by a mechanism which remains poorly understood, but which involves binding to PrP^{C} and causing this protein to convert to the PrP^{Sc} state (Fig. 12.1) (Kocisko et al. 1994; Prusiner 1997).

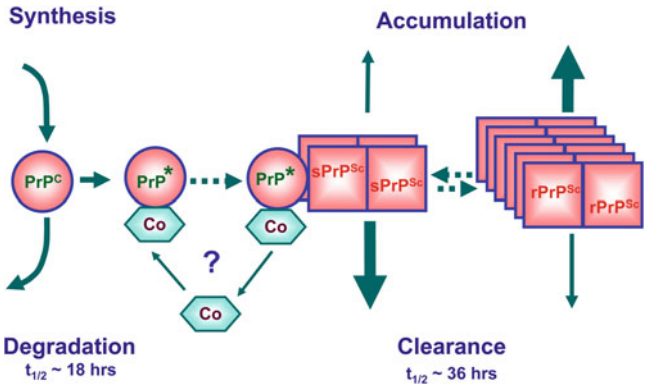

Fig. 12.1 Schematic reaction coordinates of $sPrP^{Sc}$ and $rPrP^{Sc}$ formation and accumulation. Different conversion and clearance rates of PrP^{Sc} dictate the speed of accumulation and thus incubation time in particular prion isolates

The first suggestion that properties of PrP^{Sc} might be distinct in various strains of prions arose from an analysis of two prion isolates from mink that had been passaged in Syrian hamsters and labeled drowsy (DY) and hyper (HY) according dominant clinical symptoms (Bessen and Marsh 1992, 1994). The more pronounced resistance of HY PrP^{Sc} to limited proteinase K digestion and distinct sedimentation velocity suggested dissimilar physical properties of PrP^{Sc}, but the results did not correlate with other isolates that produced similar incubation times and indistinguishable patterns of PrP^{Sc} on WBs (Scott et al. 1997). Only when prion strains generated de novo in humans with inherited prion diseases were passaged in Tg(MHu2M) mice could an argument be made for the distinctive conformation or ligands of PrP^{Sc} present in different prion strains (Telling et al. 1996; Prusiner 1997). These studies were fortuitous in the sense that fCJD(E200K) and fatal familial insomnia (FFI) produced different sizes of $rPrP^{Sc}$ fragments after limited proteinase K digestion on WBs.

The WB-based studies of PrP^{Sc} were limited to the most protease-resistant fraction of PrP^{Sc}. It has also been difficult to analyze low levels of PrP^{Sc} in the presence of high levels of PrP^{C}. Moreover, the limited digestion by proteinase K resulting in either 19- or 21-kDa bands after deglycosylation of PrP 27-30 could not explain the broad biological diversity observed in more than 30 rodent-adapted prion strains in bioassays. In response to these problems, we developed a rapid, specific, and highly sensitive method for the detection and conformational characterization of PrP^{Sc} designated as conformation-dependent immunoassay (CDI) (Safar et al. 1998). After assay calibration with recombinant PrP that has refolded into different conformations, we could distinguish α-helical, β-sheet, and random coil conformations of PrP, either alone or in a mixture. Thus, the assay enabled us to directly measure the amount of PrP^{Sc} in brain homogenates without prior digestion with proteinase K to eliminate PrP^{C}. The assay is conformation sensitive, and with selective precipitation of PrP^{Sc} before differential immunoassay, PrP^{Sc} could be measured in a sandwich

format in the presence of ~10,000-fold excess of PrP^C with a sensitivity similar to that of bioassays (Safar et al. 1998, 2002, 2005b, 2008; Kim et al. 2011).

The CDI led to the discovery of a variable fraction of pathogenic prion protein that is actually protease sensitive ($sPrP^{Sc}$) (Fig. 12.1) and allowed us to differentiate all eight strains examined by differently exposed epitopes, response to limited digestion with proteinase K, and stability in a chaotrope guanidine hydrochloride (Gdn HCl) (Safar et al. 1998). Thus, our data provided compelling evidence that eight different strains passaged in the same host (Syrian hamsters) possess at least eight distinct conformations. The differences in conformation of PrP^{Sc} detected by CDI in different prion strains in brain homogenates suggested two markedly distinct conformational mechanisms responsible for propagation of different prion characteristics. Under one possibility, each strain would be encoded by the PrP^{Sc} molecules in a definite number of conformations, and a specific mixture (ratio) of the same building blocks would replicate itself in the next passage. The second possibility is that each strain characteristic is encoded in a unique conformer of PrP^{Sc}, which then replicates with a high degree of fidelity and thus reproduces the strain characteristics.

Thus, in addition to a structure for PrP^C that is distinct from PrP^{Sc}, our data on prion strains in Syrian hamsters suggested that there may be several PrP^{Sc} conformers with distinct stabilities (energies) (Fig. 12.4) (Shirley 1995). This hypothesis represents an obvious departure from earlier work demonstrating that most proteins had a single folded structure that was uniquely encoded in the sequence (Anfinsen 1973). What is the structural basis of these alternative PrP^{Sc} conformers? Work on diphtheria toxin identified distinct crystal forms that displayed different tertiary and quaternary structures for a single polypeptide sequence (Bennett et al. 1995). To describe this observation, the notion of domain swapping was introduced whereby a region of one monomer displaced the corresponding region in another monomer to create an interlocking molecular handshake (Cohen and Prusiner 1998). This phenomenon has now been observed in a variety of other protein structures with the swapped elements as small as an isolated α-helix or β-strand and as large as an entire folded domain. We suspect that a similar phenomenon may be responsible for prion strains. The early experimental data obtained with infrared spectroscopy or with mass spectroscopy after hydrogen/deuterium exchange (H/X MS) confirm the conformational plasticity of PrP^{Sc} (Cobb and Surewicz 2009; Jones and Surewicz 2005; Caughey et al. 1998). In fact, conformational polymorphism (i.e., ability to form different strains) appears to be a general feature of amyloids and was observed, for instance, in fibrils formed by Aβ peptide associated with Alzheimer's disease (Paravastu et al. 2008; Petkova et al. 2002).

The data also argue that PrP^{Sc} must act as a template in the replication of nascent PrP^{Sc} molecules. It seems likely that the binding of PrP^C or a metastable intermediate PrP* (Figs. 12.1 and 12.4) (Safar et al. 1994) constitutes the initial step in PrP^{Sc} formation and that this is also the rate-limiting step in prion replication (Safar et al. 1998; Kaneko et al. 1997; Cohen and Prusiner 1998; Prusiner et al. 1998). The finding that the rate of PrP^{Sc} amplification by PMCA varies considerably for different murine strains supports the view that PrP^{Sc} structure is likely rate determining also in vivo (Karapetyan et al. 2009). However, the rate of PrP^{Sc} synthesis must also

reflect the activation energy required for the conversion process and thus is likely a function of both the conformation of the PrP^{Sc} multimer, which is believed to be strain dependent, and of the conformation of the PrP^{C} serving as substrate (Fig. 12.4). The conformational stability of PrP^{C} may depend on posttranslational modifications of PrP such as glycosylation or on association with cellular components which, by favoring certain PrP conformations, could promote preferential propagation of particular strains in different cells. The remarkable affinity of PrP^{C} for nucleic acids (King et al. 2007) and the requirement for polyanions in the PMCA reaction using purified PrP^{C} as substrate (Deleault et al. 2005) together support the view that cell components other than PrP^{C} may play an auxiliary role in prion strain replication (Geoghegan et al. 2007). Thus, the optimal conversion process of different prion strains might require different cofactors, and it is likely that the cofactor content or structure in a particular cell type may contribute to its capacity for propagating a particular strain (Fig. 12.1).

12.6 Human Prion Strains

Although remarkable progress has been made in understanding the pathology, biochemistry, and structure of rodent-adapted prion strains (Prusiner et al. 2004b; Caughey et al. 2009; Cobb and Surewicz 2009; Morales et al. 2007; Watts and Westaway 2007; Telling 2008), understanding of the molecular basis of human prion diseases has lagged behind. The human prion diseases are more complex, and a single pathologic process may present as a sporadic, genetic, or infectious illness (Prusiner 2004). The most common human prion disease is sporadic Creutzfeldt–Jakob disease (sCJD), accounting for ~85% of cases. Although sCJD was shown to be transmissible to nonhuman primates 40 years ago (Gibbs et al. 1968; Brown et al. 1994), the origin, pathogenesis, and the number of human prion strains causing the disease remain unknown.

Lack of progress in the area of human prions stems from three barriers. First, these diseases present greater variability on complex genetic background; second, experiments with human material are prohibitive; and finally, relatively few investigators focus on human prion diseases. Nevertheless, researchers today generally agree that the genotype at codon 129 of the chromosomal gene PRNP underly the susceptibility to prions and to some degree the phenotypes of diseases (Gambetti et al. 2003; Bishop et al. 2010; Giles et al. 2010). In contrast to the experiments with laboratory rodent prion strains, in which the digestion of brain PrP^{Sc} 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 ($rPrP^{Sc}$) found in sCJD brains are easily distinguishable on Western blot (WB) (Gambetti et al. 2003; Telling et al. 1996; Collinge et al. 1996; Parchi et al. 1997; Wadsworth et al. 1999; Zou et al. 2003) (Fig. 12.2).

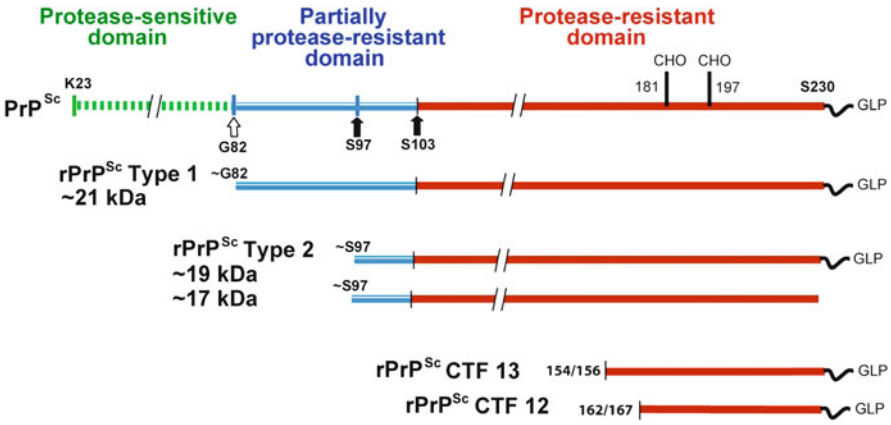

Fig. 12.2 Schematic representation of sCJD PrP^{Sc} and outline of classification of WB fragments of $rPrP^{Sc}$ (PrP 27-30) (Gambetti et al. 2003; Parchi et al. 1997, 1999; Zou et al. 2003). Major cleavage sites by PK are indicated by *arrows*; *GLP* glycolipid; *CHO* N-linked complex glycosylation chains

Although the disease phenotypes of patients with sCJD are remarkably heterogeneous, the WB findings together with human PRNP gene polymorphism led Parchi, Gambetti, and colleagues to posit a clinicopathologic classification of sCJD into five or six subtypes. Importantly, it has been shown that the WB characteristics of PrP^{Sc} breed true upon transmission to susceptible transgenic mice and guinea pigs (*Cavia porcellus*) (Gambetti et al. 2003; Telling et al. 1996; Safar et al. 2011; Parchi et al. 1997) (Fig. 12.2). Subsequently, Collinge and collaborators (Collinge et al. 1996; Collinge and Clarke 2007; Wadsworth et al. 1999; Hill et al. 1997) introduced an alternative classification of the PrP^{Sc} types and their pairing with CJD phenotypes that differed from the previous one in two aspects (a) it recognized three different electrophoretic mobilities of PrP^{Sc} and (b) differentiated distinct glycoform ratios in PrP^{Sc} (Collinge and Clarke 2007).

Because the disease duration and phenotypes associated with 21-kDa fragments of unglycosylated PrP^{Sc} (type 1) frequently differ from the 19-kDa fragments of PrP^{Sc} (type 2) (Fig. 12.3) (Gambetti et al. 2003; Telling et al. 1996; Parchi et al. 1997; Monari et al. 1994), these findings argue that the PrP^{Sc} type may represent another modifier of the phenotype in human prion diseases. Consequently, WB-based clinicopathologic classifications became useful tool in studies of prion pathogenesis in transgenic mice models of human prion diseases and in human brains (Telling et al. 1996; Collinge and Clarke 2007). Because two distinct PK cleavage sites in PrP^{Sc} types 1 and 2 most likely originate from different conformations, some investigators contend that PrP^{Sc} types 1 and 2 code distinct prion strains (Parchi et al. 1996; Telling et al. 1996; Collinge et al. 1996; Monari et al. 1994). However, the findings of the co-occurrence of PrP^{Sc} types 1 and 2 in 40% or more of sCJD cases suggested that the originally observed differences were quantitative rather than

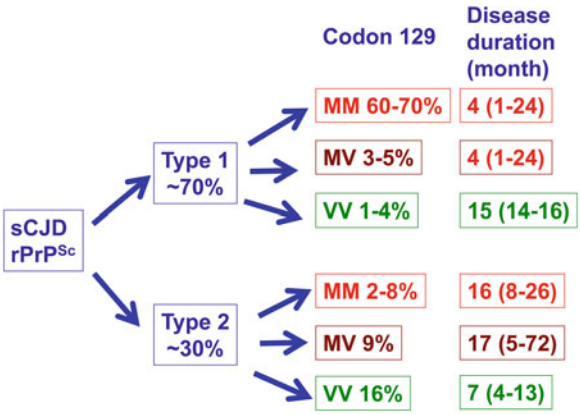

Fig. 12.3 Impact of the polymorphism in codon 129 of the PRNP gene and WB pattern of PrP^{Sc} on the susceptibility to sCJD and duration of the disease. The *first number* indicates the mean duration of the disease; the range is in the *parenthesis* (Gambetti et al. 2003; Parchi et al. 1997, 1999; Zou et al. 2003)

qualitative (Puoti et al. 1999; Kovacs et al. 2002; Head et al. 2004; Lewis et al. 2005; Schoch et al. 2006; Cali et al. 2009). Additionally, the extensive phenotypic heterogeneity of sCJD, along with a growing number of studies including bioassays, all suggests that the range of prions causing sCJD exceeds the number of categories recognized within the original WB-based clinicopathologic schemes (Safar et al. 2005b; Uro-Coste et al. 2008; Polymenidou et al. 2005). Finally, up to 90% of PrP^{Sc} is protease sensitive (s), and the conformation and the role of this fraction in the pathogenesis of the disease are unknown and remain a subject of speculation (Safar et al. 2005b, c; Cronier et al. 2008) because it is destroyed by proteinase K treatment, which is necessary to eliminate PrP^{C} (Safar et al. 2005b). Cumulatively, no direct structural data are available for sCJD brain PrP^{Sc} beyond the evidence that it is variably resistant to proteolytic digestion.

To determine the conformational range and strain-dependent structural characteristics of sCJD PrP^{Sc} in patients who were homozygous for codon 129 of the PRNP gene and thus advance our understanding of the molecular pathogenesis of human prion diseases, we introduced the conformation-dependent immunoassay (CDI) (Safar et al. 1998, 2002, 2005b). The conformational stability of the protein in a denaturant such as Gdn HCl (Shirley 1995) is reflecting the original conformation of the protein. If the protein has the same amino acid sequence, the difference in stability indicates the difference in conformation. Thus, even relatively minute variations in a protein structure can be determined. Using this concept, we developed conformational stability assay in which PrP^{Sc} is first exposed to denaturant Gdn HCl and then to europium-labeled mAb against the epitopes hidden in the native conformation (Safar et al. 1998). With sequentially increasing concentration of Gdn HCl, PrP^{Sc} dissociates and unfolds from native β-sheet-structured aggregates and more epitopes become available to antibody binding. Because PrP^{Sc} is insoluble oligomer and denaturation of this protein is irreversible in vitro, the Gibbs free energy change (ΔG) of PrP^{Sc} cannot be calculated (Safar et al. 1994). Therefore, we introduced instead the Gdn HCl value found at the half-maximal denaturation ($[GdnHCl]_{1/2}$) as a measure of the relative conformational stability of PrP^{Sc}.

The differences in $[GdnHCl]_{1/2}$ reveal evidence of distinct conformations of PrP^{Sc} (Safar et al. 1994, 1998; Shirley 1995).

The process of disaggregation and unfolding of PrP^{Sc} in the presence of increasing concentration of Gdn HCl has been described as follows:

$$\left[\mathrm{Pr\,P^{Sc}}\right]_n \rightarrow \left[\mathrm{sPrP^{Sc}}\right]_n \rightarrow \mathrm{iPrP} \rightarrow \mathrm{uPrP}$$

where $[PrP^{Sc}]_n$ are native aggregates of PrP^{Sc}, $[sPrP^{Sc}]_n$ are soluble protease-sensitive oligomers of PrP^{Sc}, iPrP is an intermediate, and uPrP is completely unfolded (denatured) PrP (Safar et al. 1993, 1994, 2011; Tzaban et al. 2002; Safar 2012). Since CDI is not dependent on protease treatment, it allowed us to address fundamental questions concerning the concentration and conformation of different isoforms of sCJD PrP^{Sc}, including protease-sensitive (s) and protease-resistant (r) PrP^{Sc} (Kim et al. 2011; Safar 2012). Consequently, the CDI monitors the global transition from native aggregates to fully denatured monomers of PrP^{Sc}. In contrast, the WB-based techniques monitor either the partial solubilization of PrP^{Sc} (Pirisinu et al. 2011) or conversion of $rPrP^{Sc}$ to protease-sensitive conformers (Peretz et al. 2001) after exposure to denaturant. Therefore, stability data on protease-sensitive oligomers and intermediates of PrP^{Sc} cannot be obtained with WB and may lead to some markedly different values (Choi et al. 2011).

We found with CDI a remarkable heterogeneity of PrP^{Sc} conformations within sCJD patients homozygous for codon 129 polymorphism of the PRNP gene and a range corresponded to that of stabilities found in ~30 distinct strains of natural and de novo laboratory rodent prions that were examined so far (Safar et al. 1998; Peretz et al. 2001; Kim et al. 2011; Colby et al. 2010). The unexpected differential effect of PK treatment with increasing stability of type 1 and decreasing stability of type 2 PrP^{Sc}(129M) suggests that in contrast to type 1, the protease-resistant core of type 2 is less stable (Fig. 12.4). The increased frequency of exposed epitopes and decreased stability in type 2 PrP^{Sc} after PK treatment (Kim et al. 2011) are counter-intuitive 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 PrP^{Sc} resulted in less stable conformation with more exposed 108–112 epitopes, or that the ligand protecting the 108–112 epitopes and stabilizing the PrPSc was removed by PK. Whether the epitopes' hindrance in undigested PrP^{Sc} is the result of lipid, glycosaminoglycan, nucleic acid, or protein binding to the conformers unique to the MM2 sCJD PrP^{Sc} 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, our findings indicate that sCJD PrP^{Sc} exhibits extensive conformational heterogeneity and suggest that a wide spectrum of sCJD prions cause the disease (Safar 2012). Whether this heterogeneity originates in a stochastic misfolding process that generates many distinct self-replicating conformations (Collinge and Clarke 2007; Prusiner 2001) or in a complex process of evolutionary selection during development of the disease (Li et al. 2010) remains to be established (Kim et al. 2011; Safar 2012).

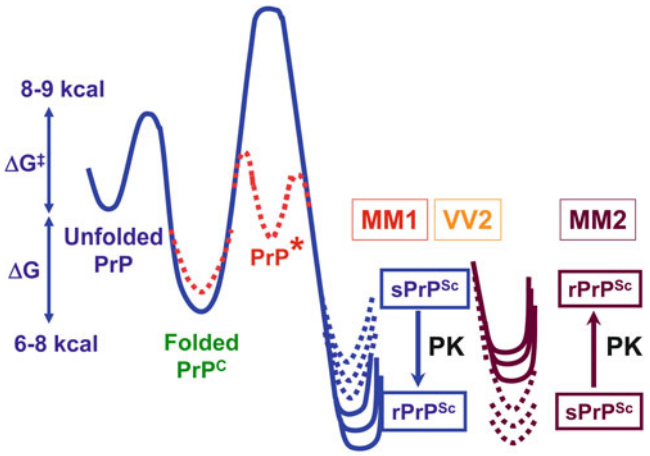

Fig. 12.4 Schematic representation of the energy landscape of different PrP^{Sc} conformers in sCJD and the impact of protease treatment. Distinct conformers within the same WB type are depicted with *multiple lines*

Despite the inevitable influence of the potential difficulties in evaluating initial symptoms and variable genetic background, our recent data indicate that the levels as well as stability of $sPrP^{Sc}$ are a good predictor of the progression rate in sCJD (Kim et al. 2011). The disease progression rate and incubation time jointly represent replication rate, propagation, and clearance of prions from the brain (Prusiner et al. 2004a; Safar et al. 2005c). Therefore, the correlations among the levels of $sPrP^{Sc}$, the stability of $sPrP^{Sc}$, and the duration of the disease all indicate that $sPrP^{Sc}$ conformers play an important role in the pathogenesis. When $sPrP^{Sc}$ is less stable than $rPrP^{Sc}$, the difference in stability correlates with less accumulated $sPrP^{Sc}$ and shorter duration of the disease. An opposite effect is observed when sPrP conformers are more stable than $rPrP^{Sc}$—more accumulated $sPrP^{Sc}$ and extended disease duration (Fig. 12.4) (Kim et al. 2011). These observations parallel the experiments on yeast prions and suggest that the stability of misfolded protein is inversely related to the replication rate (Kim et al. 2011; Tanaka et al. 2006). Thus, the data from both yeast and human prions lead to the hypothesis that the less stable prions replicate faster by exposing more available sites for growth of the aggregates. Although the modulating effect of prion clearance in the mammalian brains is likely (Safar et al. 2005c), faster prion replication leads to shorter incubation time and faster progression of the disease.

12.7 Outlook

The continuing mystery surrounding replication of the PrP^{Sc} conformer poses a fundamental challenge in modern biology, and important questions regarding prion strains remain to be answered. For example, is each strain composed of a unique

conformer or of a spectrum of conformations, which may shift by selection or conformational evolution? Additionally, the conformational concept of prion strain replication raises the question of which conformational features of PrP^{Sc} are important for replication and which determine clearance. Although there is now convincing evidence that the PrP^{Sc} conformation of distinct strains is different, it is not known to what extent the conformation or replication rate of different conformers might depend on factors other than conformation of the PrP, for example, the nature of the glycans or additional cell-derived ligands (cofactors). An attractive experiment would be to obtain large quantities of highly purified PrP^{Sc} from a single cell line, infected separately with several different prion strains; determine the glycans carried by each strain-associated PrP^{Sc}; and search for associated molecules, such as small RNAs or other cell components. Finally, the deepest insight will be gained once the three-dimensional structure of PrP^{Sc} can be determined at high resolution, currently a still formidable task.

Acknowledgments This work was supported by grants from NIA (AG-14359), NINDS (NS074317), CDC (UR8/CCU515004), and the Charles S. Britton Fund.

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