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Editor
Prof. Dr. Jörg Tatzelt
Ludwig Maximilians University Munich
Adolf Butenandt Institute &
German Center for Neurodegenerative Diseases (DZNE)
Schillerstr. 44
80336 Munich
Germany
joerg.tatzelt@med.uni-muenchen.de

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Volume Editor

Prof. Dr. Jörg Tatzelt

Ludwig Maximilians University Munich
Adolf Butenandt Institute &
German Center for Neurodegenerative Diseases (DZNE)
Schillerstr. 44
80336 Munich
Germany
joerg.tatzelt@med.uni-muenchen.de

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The series *Topics in Current Chemistry* presents critical reviews of the present and future trends in modern chemical research. The scope includes all areas of chemical science, including the interfaces with related disciplines such as biology, medicine, and materials science.

The objective of each thematic volume is to give the non-specialist reader, whether at the university or in industry, a comprehensive overview of an area where new insights of interest to a larger scientific audience are emerging.

Thus each review within the volume critically surveys one aspect of that topic and places it within the context of the volume as a whole. The most significant developments of the last 5–10 years are presented, using selected examples to illustrate the principles discussed. A description of the laboratory procedures involved is often useful to the reader. The coverage is not exhaustive in data, but rather conceptual, concentrating on the methodological thinking that will allow the non-specialist reader to understand the information presented.

Discussion of possible future research directions in the area is welcome.

Review articles for the individual volumes are invited by the volume editors.

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Preface

Prion diseases are a group of transmissible neurodegenerative disorders including Creutzfeldt-Jakob disease (CJD) and Gerstmann-Sträussler-Scheinker syndrome (GSS) in humans, scrapie in sheep and goat, bovine spongiform encephalopathy (BSE) in cattle and chronic wasting disease (CWD) in deer. A hallmark of prion diseases is the formation and accumulation of an aberrantly folded protein denoted scrapie prion protein (PrP^{Sc}), which co-purifies with the infectious prion agent. Remarkably, the prion protein was found to be encoded by a host gene, which is constitutively transcribed under physiological conditions. We now know that not the expression of the prion protein itself is the disease-specific feature but rather its biophysical and biochemical properties. In contrast to the cellular isoform PrP^{C}, PrP^{Sc} is insoluble in non-ionic detergents, partially resistant to proteolytic digestion and characterized by a high content of β-sheet secondary structure. Furthermore, PrP^{Sc} has the propensity to form amyloid plaques in the diseased brain.

Formation of aberrantly folded protein conformers is also a hallmark of other neurodegenerative disease in humans, including Alzheimer's disease, Parkinson's disease, and polyglutamine disorders. As an unprecedented feature of prion diseases, however, the formation of non-native PrP conformers is causally linked not only to neurodegeneration but also to the formation of infectious particles. Another unique hallmark of prion diseases is that they naturally occur in some other mammalian species. Moreover, in contrast to other neurodegenerative diseases rodent models of prion diseases are available that faithfully recapitulate all aspects of human prion diseases and allow to specifically study either neurotoxic or infectious properties of PrP conformers. In combination with robust cell culture models and *in vitro* approaches it has thus been possible to decipher pathophysiological mechanisms implicated in both the formation of infectious and neurotoxic PrP conformers, a prerequisite to explore prophylactic or therapeutic strategies. While prion diseases in humans are extremely rare, the established models may help to provide new insight into common mechanisms underlying the toxic activity of misfolded protein conformers of different origins. In particular, it is plausible to assume that certain cellular components and pathways, for example the cellular

stress response, the ubiquitin-proteasome system, or molecular chaperones, play important roles in the pathogenesis of diverse protein misfolding diseases.

In this volume we have assembled a set of chapters highlighting various aspects of prion diseases in humans and animals which show the intriguing features of both physiological and pathogenic PrP conformers. In addition, a wide spectrum of methodological approaches are described, ranging from *in silico*, chemical, and *in vitro* approaches to animal models, to illustrate the elaborate and extensive technology established in the prion field, allowing the analysis of disease mechanisms and the development of diagnostic and therapeutic strategies. Finally, the reviews on shadoo, doppel and yeast prions emphasize that certain features and activities of the prion protein can also be found in other proteins and that the phenomenon of self-replicating protein conformers can be of broader biological significance.

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Genetics of Prion Disease

Sarah Lloyd, Simon Mead, and John Collinge

Abstract Prion diseases or transmissible spongiform encephalopathies (TSEs) are neurodegenerative disorders of humans and animals for which there are no effective treatments or cure. They include Creutzfeldt-Jakob disease (CJD) in humans and sheep scrapie, bovine spongiform encephalopathy (BSE) and chronic wasting disease (CWD) in cervids. The prion protein (PrP) is central to the disease process. An abnormal form of PrP is generally considered to be the sole or principal component of the infectious agent and a multimeric isomer (PrP^{Sc}) is deposited in affected brains. Inherited prion diseases are caused by over 30 mutations in the prion protein gene (*PRNP*) and common polymorphisms can have a considerable affect on susceptibility and phenotype. Susceptibility and incubation time are also partly determined by other (non-*PRNP*) genetic modifiers. Understanding how these other genes modify prion diseases may lead to insights into biological mechanisms. Several approaches including human genome wide association studies (GWAS), mouse mapping and differential expression studies are now revealing some of these genes which include *RARB* (retinoic acid receptor beta), the E3 ubiquitin ligase *HECTD2* and *SPRN* (Shadoo, shadow of prion protein gene).

Keywords Creutzfeldt-Jakob disease (CJD) · Incubation time · Inherited prion disease (IPD) · Polymorphism · Prion · Prion protein gene (*PRNP*) · Susceptibility

Contents

S. Lloyd, S. Mead, and J. Collinge (✉)
MRC Prion Unit and Department of Neurodegenerative Diseases, UCL Institute of Neurology, London WC1N 3BG, UK
e-mail: j.collinge@prion.ucl.ac.uk

1 Introduction

Prion diseases, also known as transmissible spongiform encephalopathies, are a group of fatal neurodegenerative disorders affecting both humans and animals. Human prion diseases include Creutzfeldt-Jakob disease (CJD), Gerstmann–Sträussler–Scheinker syndrome (GSS), fatal familial insomnia (FFI) and kuru. The best known animal diseases include sheep scrapie, bovine spongiform encephalopathy (BSE) and chronic wasting disease (CWD) in cervids. Uniquely, prion disease aetiology can be described as inherited (familial), sporadic (unknown cause) or acquired (transmission of infectious agent). The classical neuropathology of these diseases includes spongiform change, gliosis and an accumulation of an abnormal multimeric isomer (PrP^{Sc}) of the host-encoded cellular prion protein (PrP^{c}). PrP plays a central role in the disease process and its abnormal form is generally considered to be the sole or principal component of the infectious agent.

The prion protein gene (*PRNP* – human, *Prnp* – mouse) is found on chromosome 2 in mouse and chromosome 20 in humans. The human gene comprises 2 exons (3 in mouse) with the open reading frame, encoding the 253 amino acid human protein (PrP), entirely within the larger second exon [1]. The mature prion protein has a signal peptide cleaved from the N-terminus and a C-terminal peptide is cleaved when the protein is attached to its glycosylinositolphosphatidyl anchor. The mature cell surface protein has an unstructured N-terminal domain containing an octapeptide repeat region, and a predominantly alpha-helical C-terminal domain with two variably utilised glycosylation sites.

Mammalian genetics has had a profound impact on our current understanding of prion disease pathogenesis. Incubation time studies using mouse-adapted sheep scrapie and study of several human inherited prion diseases allowed the mapping of alleles to the region of the prion protein gene in both species. Mutations in the prion protein gene (*PRNP*) are the sole cause of inherited prion diseases. The generation of infectious prions by patients with these mutations remains one of the most important observations in support of the hypothesis that prion diseases do not require exogenous nucleic acids for their infectious properties, termed the protein-only hypothesis [2, 3]. Polymorphisms of human, mouse, sheep and cervid prion protein genes,

for example the common variation at codon 129 of the human prion protein gene between methionine and valine, have a profound effect on susceptibility and clinical phenotype. These historically important discoveries, more recent developments and challenges for the future genetics of prion diseases are discussed further below.

2 Inherited Prion Disease

There are now at least 30 different mutations known to cause inherited prion disease (IPD), and these are inherited as autosomal dominant traits (Fig. 1). The first to be discovered, an insertion mutation of six extra octapeptide repeats in the N-terminal domain of PrP, was described in 1989 [4], shortly followed by the discovery of the P102L mutation in GSS families [5]. There are broadly two types of mutation: alteration of the normal number of repeats in the N-terminal region of PrP (normally a nonapeptide followed by four octapeptide repeats between codons 51 and 91 of *PRNP*), and point mutations in the C-terminal portion of the protein. Clinical manifestation of IPD can be highly variable even in patients with the same mutation and within a single family [6]. Some of the inherited prion diseases have been successfully transmitted to laboratory animals, including the most prevalent 6-octapeptide repeat insertion (OPRI), P102L, D178N and E200K mutations, although transmission has not been attempted for many, and several mutations have failed to transmit [7]. Nevertheless, the fact that any germline mutation can generate an infectious entity was critical evidence in support of the protein-only hypothesis.

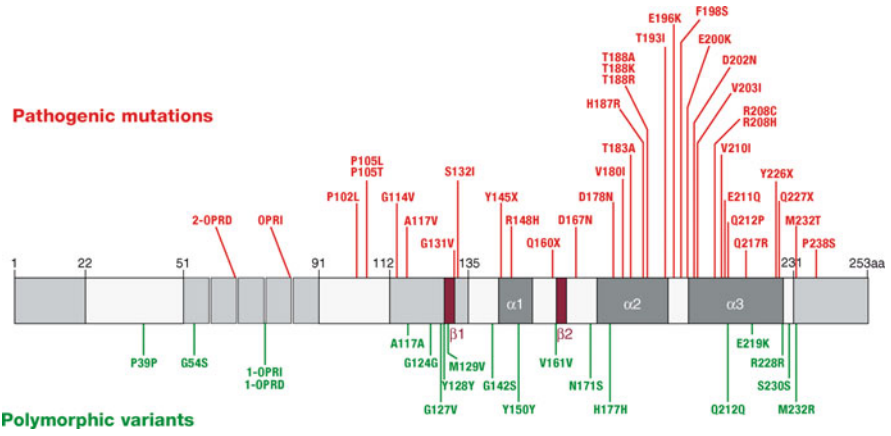

Fig. 1 Diagram of the human prion protein gene showing the octapeptide repeat region (51–91), regions of predicted secondary structure [designated α (helical) or β (sheet)], pathogenic or likely pathogenic mutations (above in *red*), non-synonymous polymorphisms and synonymous polymorphisms (below in *green*)

2.1 Octapeptide Repeat Insertion Mutations

A number of families with 6-OPRI mutations have now been described; the largest group of over 100 patients has a 144 bp insertion (6 × 24 bp repeats or 6-OPRI) [6, 8, 9]. These patients have a mean age of onset of symptoms of 35 (range 20–53) years and mean age at death of 45 (30–65) years. The clinical duration is around 10 years, but younger patients appear to have a longer clinical duration than older patients. Typical presenting clinical features are cortical cognitive deficits encompassing acalculia, language dysfunction, apraxia and memory impairment together with frontal behavioural disturbance. There is evidence of an early personality disorder that might suggest a neurodevelopmental deficit caused by the mutation (possible haploinsufficency) [6]; however, the aetiology of this clinical feature is hard to disentangle from social factors related to the impact of a particularly early-onset dementing and neurological illness in the parent.

A number of different 5-OPRI mutations are described including families from the UK, South Africa, Northern Ireland, USA and Eastern Europe [10]. The phenotypic variation is again notable in this group. The age of onset ranges from the third to the seventh decade and the duration of the illness from 4 months to 15 years. Some patients are clinically indistinguishable from sporadic CJD (sCJD) but most have a slower dementing disorder mistaken for Alzheimer's disease only later accompanied by apraxia, ataxia and sometimes myoclonus. In association with pathological evidence of prion disease, 7, 8 and 9 octapeptide repeat insertions have also been described. The clinical syndrome associated with these mutations is very similar to the 6-OPRI mutation. The neuropathology of the insertional mutations may be characterised by a stripe-like pattern of abnormal PrP deposition in the molecular layer of the cerebellum, perpendicular to the prial surface [11].

Occasionally patients with a clinical phenotype typical of sCJD have other octapeptide repeat mutations, but the exact significance of these mutations in this group is unclear in many [12]. Those involving one, two or three additional repeats may be coincidental findings, as these have been found in healthy control populations [13]. Several patients with 4-OPRI insertions have been reported associated with a late-onset, short duration clinical course, often with an absence of family history and so are often mistaken for sCJD; one possible explanation for this observation is discussed below.

In 4-, 5- and 6-OPRI diseases, the genotype at codon 129 of *PRNP* is a major determinant of age of clinical onset. In the published studies addressing this issue, the OPRI mutations are linked to methionine at codon 129, and patients therefore have either codon 129 methionine homozygous or methionine-valine heterozygous genotypes. In 5- and 6-OPRI both genotypes are fully penetrant by age 70; however, those patients with a heterozygous genotype have, on average, a later clinical onset by around 10 years. In 4-OPRI the typical disease onset is in old age. However, all or the vast majority of patients have a methionine homozygous genotype, implying that those with a heterozygous genotype at codon 129

may have a disease onset beyond a natural lifespan (i.e. non-penetrance). Although first described in the 6-OPRI disease this codon 129 modifying effect has now been seen in several other inherited prion diseases (e.g. P102L, A117V, F198S) [14, 15]. The genetic observations may be accommodated by a model of prion replication that favours homotypic protein interactions in the kinetics of prion replication [16, 17].

There are only two reports of a deletion in the repeat region (2-OPRD) in patients with CJD [18, 19]. As the 2-OPRD has not been found in healthy individuals it is likely to be a causal mutation. The 1-OPRD is an uncommon polymorphism of some healthy populations; it is not known to be a susceptibility or modifying factor [20].

2.2 Point Mutations

A large number of point mutations have been described. Several show marked ethnogeographic clustering related to the occurrence of a mutation in a founder whose descendants migrated and increased in frequency in different geographical regions in accordance with the history of human populations. Only the common mutations that illustrate some of the key genetic aspects of inherited prion diseases are described here.

The P102L mutation, described in 1989 [5], is the archetypal cause of Gerstmann–Sträussler–Scheinker disease [15]. Ataxia is therefore the commonest symptom with later or less prominent cognitive decline, leg weakness and lower limb pain. Additional features include psychiatric symptoms and pyramidal and extrapyramidal signs in a minority. Occasional CJD-like atypical patients are seen, sometimes with a striking early clinical onset; this observation may be caused by involvement of wild-type PrP in the pathologic process [21]. The neuropathology is characterised by multicentric plaques of PrP in the brainstem and cerebellum.

The D178N mutation is important in that there is some evidence that the clinical phenotype of the disease is modified by the haplotype formed with the mutation-linked allele at codon 129. FFI, the third canonical prion disease syndrome, is said to be caused when the mutation occurs on the 129 methionine allele; it was first described in Italians but has since been identified worldwide. Insomnia is the cardinal feature of the disease often preceded by lack of attentiveness. The insomnia may be masked by apparent excessive daytime sleeping as a result of lack of nocturnal somnolence. This is soon accompanied by autonomic symptoms such as hypertension, excessive sweating, evening pyrexia, salivation and impotence. Later hallucinations occur, often related to dreams, and the patients may have limb movements related to the dreaming. As the disease progresses, ataxia, pyramidal signs and myoclonus occur in many patients. Whilst this striking clinical phenotype undoubtedly occurs in 129M-178N IPD, there is not a consistent association between haplotype and phenotype, even within the same family with the same mutation, and many of the clinical features are observed in patients with other types

of prion disease. 129V-178N more typically causes a CJD-type rather than FFI clinical picture. These patients present with memory impairment often at a younger age than sporadic CJD [22].

Overall, the commonest mutation worldwide is E200K, and additionally, in certain regions, e.g. Eastern Europe, Israel, North Africa and Chile, the disease may have a similar prevalence as sporadic CJD [23]. Patients are clinically indistinguishable from those with sCJD apart from the occasional finding of a peripheral neuropathy of mixed axonal and demyelinating type [24] and seizures are more common than in sCJD [23]. E200K patients are on average slightly younger than sCJD subjects but there is great variation and examples of non-penetrance in late old age. In the majority of patients the mutation is linked to the methionine allele; in the unusual circumstance of 200K linkage to valine, patients are reported to have a longer clinical course and more ataxia.

3 Genetic Susceptibility to Acquired and Sporadic Human Prion Disease

Strong genetic susceptibility to prion disease has been clearly demonstrated in both acquired and sporadic human prion disease. A very strong and common prion disease genetic risk factor is now well known at *PRNP* codon 129, between methionine (~60% allele frequency in Europeans) and valine [16]. In European and North American healthy populations the methionine heterozygous genotype is generally the most frequent (~50% in the UK), followed by methionine homozygous (~40% in the UK), with the least common being valine homozygous genotypes (~10% in the UK). The polymorphism is found around the world, with a gradual decline in 129V frequency from that found in Europeans, moving east into Asia and south into Africa. Notable exceptions to this trend are very high 129V frequencies in the Eastern Highlands of Papua New Guinea (PNG) and in several South American populations [25].

As described above for some of the inherited prion diseases, heterozygosity at *PRNP* codon 129 is protective against prion disease, a finding which can be generally explained by heterotypic protein interactions impeding the kinetics of prion replication. Heterozygous genotypes are strongly underrepresented in sCJD [16], and when described are associated with atypical clinical and pathological phenotypes. Heterozygosity is also associated with a long incubation period in iatrogenic CJD [26] (acquired by dura mater transplantation or cadaver-derived growth hormone therapy). Perhaps the most striking association however is that all confirmed and tested vCJD patients are methionine homozygous, representing the strongest association of a common genotype with any disease [27]. Recently a single clinically-suspected vCJD patient had a methionine-valine heterozygous genotype [28]; however, no molecular investigations were conducted to provide a definitive confirmation of diagnosis. The association of codon 129 genotype with vCJD requires more explanation than simply the resistance provided by heterozygosity, as to date there have been no valine homozygous patients. However, evidence from transgenic models suggests

PrP 129V may not be able to adopt the molecular conformation associated with the vCJD prion strain [29]. The observations are best interpreted in the context of the conformational selection hypothesis of prion replication [17, 30]. According to this model the 129 polymorphism may also affect susceptibility by determining which prion strains are thermodynamically permissible or favoured in a given host. An understanding of susceptibility conferred by *PRNP* codon 129 has had a major impact on prion biology [7]. The strong impact of the codon 129 genotype across all human prion disease categories is an important precedent for research work focussed on discovery of other genetic risk factors.

Kuru is a fatal subacute neurodegenerative disease restricted to the Fore people and their immediate neighbours in the Eastern Highlands of PNG. Its route of transmission was oral, through the consumption of infected brain and other material from dead patients at mortuary feasts [31]. Kuru provides the main experience of an epidemic human prion disease, and it is one that is nearly complete. There is a remarkable range of possible incubations in orally acquired human prion infection, from around 5 to over 50 years [32]. As in other types of human prion disease, *PRNP* codon 129 is a strong susceptibility factor. Codon 129 heterozygosity strongly associates with long-term survival after exposure to kuru prions [25], and with long incubation time in kuru patients [32]. Recently, a novel *PRNP* variant at codon 127 (glycine to valine) was found exclusively in villages in the Purosa valley of the kuru region. This variant was present in half of the otherwise susceptible 129MM women from the region of highest exposure to kuru prions. Although this allele is common in the area with the highest incidence of kuru, it was not found in patients with kuru and in unexposed population groups worldwide. The 127V polymorphism has been proposed to be a new acquired prion disease resistance factor that increased in frequency in the Fore due to Darwinian selection during the kuru epidemic [33].

In the neighbouring genetic region to *PRNP* on chromosome 20, several studies have addressed the existence of additional genetic risk factors beyond codon 129 [34, 35]. The hypothesis driving this work is that genetic variation in regulatory regions might lead to altered expression of the gene, which is known from transgenic mouse experiments to be an important determinant of incubation time [36]. These studies have generally been positive in sporadic CJD, although the strength of the association of variants both up- and downstream of the gene is very modest. In particular, a haplotype designated "B" in 2001 has been identified as a risk factor for sporadic CJD in UK and German populations [34, 35].

One of the most important developments in genetic technology has been the advent since 2005 of commercial gene-arrays able to genotype accurately hundreds of thousands of common single nucleotide polymorphisms (SNPs) in human populations. The presence of an allele often predicts neighbouring alleles on a chromosome because of linkage disequilibrium; as a result, a large proportion of genomic common genetic variation can be captured with this technology, enabling genome wide association studies (GWAS). These studies simply compare case sample allele counts with the healthy population by a chi-squared test applied across hundreds of thousands of SNPs. GWAS studies are only powerful enough to identify modest risk factors when conducted with many thousands of patient and control samples,

which is clearly challenging in rare conditions like prion disease. However, there have been several notable exceptions to this rule, with strong genetic risk factors discovered with small GWAS samples [37]. In the circumstance of vCJD, despite the necessarily small sample, a successful study was favoured by an extremely well-defined clinical phenotype; only around 170 patients have developed the disease despite the exposure of almost the entire UK population. One interpretation of the low disease incidence is that these individuals might represent extremes of a spectrum of population susceptibility or incubation period which is known from mouse studies (see below) to be partly determined by genetic modifiers.

In order to identify strong human prion disease modifier genes, a GWAS of vCJD has been done. As a very large number of independent risk factors are being tested in these studies the statistical thresholds required to claim success are necessarily high (generally $p < 5 \times 10^{-8}$) and these "hits" need to be replicated in independent samples. Unsurprisingly in vCJD the *PRNP* locus was strongly associated with risk across several markers and all categories of prion disease; the best single SNP association in vCJD was with SNP rs6107516 at $p = 2.5 \times 10^{-17}$, a remarkably significant finding given that the sample was very small. A two-SNP combination (haplotype) formed by rs6031692 and rs6107516 at *PNRP*, had an association of $p = 1 \times 10^{-24}$. These SNPs were strongly linked to codon 129, but the entire association at the locus could not be explained by codon 129, suggesting additional but modest genetic risk factors at the locus. Aside from *PRNP*, an association upstream of *RARB* (the gene that encodes retinoic acid receptor beta) was the top non-*PRNP* association in this study, on the borderline of the threshold required for genome-wide significance ($p = 1.9 \times 10^{-7}$). A similar association at the *THRB-RARB* locus was found in a small sample of patients with iatrogenic CJD ($p = 0.030$) but not in patients with sporadic CJD (sCJD) or kuru. An association with acquired prion disease, including vCJD ($p = 5.6 \times 10^{-5}$), kuru incubation time ($p = 0.017$), and resistance to kuru ($p = 2.5 \times 10^{-4}$), in a region upstream of *STMN2* (the gene that encodes SCG10) was also detected in this study.

One of the major limitations of GWAS is that the technique is designed to identify common genetic variation as a risk factor. As is clear from the mutational series of *PRNP*, a rare amino acid change in PrP is enough to cause a fully penetrant and early onset disease. The possibility that rare mutations/variants in other genes are strong risk factors remains unassessed by current GWAS. Analysis of the candidate gene Shadoo "shadow of prion protein gene" (*SPRN*) may provide a precedent for future studies [38]. In this work two vCJD patients were found to have a single frameshift mutation of this gene, likely to be a functionally null allele as the frameshift occurred near to the 5′ end of the open reading frame. Frameshifts were not found in over a thousand control samples or sporadic CJD patients. A role for *SPRN* in human prion pathobiology has been suggested by several studies [39]. The technology for sequencing multiple regions of the genome simultaneously is developing rapidly. Rare structural variants (such as deletions, duplications, inversions) may also be assessed by current technologies.

Although significant progress has been made in discovering human susceptibility factors, the use of animal models has played an important role in understanding

the role of *Prnp* in prion disease and they remain central to further gene discovery and hypothesis testing. The validity of animal models in prion disease and their integration with human studies – animals are susceptible to the human pathogen – is a particular strength that counterbalances the challenges of working with a rare disease and small samples. An important precedent in this respect is the discovery of the first non-*PRNP* mouse and human susceptibility gene for acquired prion disease, *HECTD2*, which is discussed below.

4 The Mouse as a Model of Human Prion Disease

Mouse models have long been one of the favourite tools of the geneticist. While technological advances now make direct studies of the human genome possible, the mouse still has many advantages to offer. This is especially true for prion diseases. Uniquely amongst neurodegenerative diseases, the mouse faithfully recapitulates human pathology. Indeed, it could be argued that these are not really models at all, since wild type laboratory mice are naturally susceptible to these diseases. Comparative genomics suggests that the mouse and human genome is similar enough that genes discovered through mouse studies are also likely to be present in humans and, more importantly, the same biochemical pathways and cellular mechanisms exist in both. For rare diseases, mice can also provide the large numbers required to design sufficiently powered studies to identify the genes of interest. Mouse studies also offer access to the full range of tissues at all time points throughout disease pathogenesis with the added advantage that, in most models, onset of disease can be accurately predicted. When a candidate gene has been identified, mice provide the ideal background for hypothesis testing as a range of models, from over-expressing transgenics to knockouts, can be used to determine the effect on prion pathology.

5 Incubation Time

Prion infections in all mammals are characterised by prolonged, clinically silent, incubation periods. In experimental transmissions this is measured from the time of inoculation to the onset of diagnostic clinical signs of disease. For transmissions of mouse-adapted prions to other mice, 100% of animals are susceptible and develop clinical prion disease. Incubation time is therefore considered a suitable phenotype to model some aspects of human disease susceptibility. Under standardised experimental conditions, incubation times for a given mouse strain are remarkably predictable with reproducible means and small standard deviations. Many experimental factors have been shown to influence incubation time including infectious dose and route of inoculation. Direct administration of prions into the brain generally results in shorter incubation times compared to peripheral or oral dosing (not necessarily true for “lymphotrophic” strains). The source of infectious prions

can also have a profound influence on incubation time. Primary passage of prions into a new species results in a much longer incubation time than is seen on second and subsequent passages in the same species. This extended incubation time on primary passage is known as a species or transmission barrier. Prion strain also influences incubation time. Indeed, classical definitions of strains include their characteristic incubation times in panels of inbred mice [40]. Transgenic and knockout mouse models have shown that expression levels of host PrP^c can significantly influence incubation time. *Prnp* knockout mice ($Prnp^{0/0}$) are unable to propagate prions and never develop disease while hemizygous mice ($Prnp^{+/0}$) develop disease but with an extended incubation time as compared to their wild type littermates [36]. Conversely, transgenic mice over-expressing PrP^c have shorter incubation times showing an inverse correlation between the level of PrP^c and incubation time [36, 41]. When experimental conditions are kept constant, the main determinant of incubation time is host genetic background.

6 Role of *Prnp* in Incubation Time

The role of host genes in determining incubation time has been known for decades. In early experiments in 1964, Dickinson and MacKay challenged different inbred mice with mouse-adapted scrapie and identified a mouse strain (VM/Dk) with an unusually long incubation time [42]. Subsequent work surveyed a larger range of inbred lines of mice and showed that each mouse strain has a characteristic incubation time for a defined prion strain [43–46]. Broadly, incubation times were classed into two groups where a "short" incubation time is 100–200 days and a "long" incubation time is greater than 255 days [46]. Classical mouse crosses were carried out by several investigators to elucidate the genetic basis of this split [43, 44, 47]. They concluded that incubation time was determined by two alleles of a single gene. The gene was named respectively *Sinc* or *Prni* although they were generally considered to be the same locus. The alleles of *Sinc* were described as *s7* and *p7* and the alleles of *Prni* were *N* and *I* both encoding "short" and "long" incubation times, respectively. Following the cloning of *Prnp* on mouse chromosome 2 it was proposed that *Sinc*/*Prni* was either *Prnp* itself or very closely linked; however the experiments done at this time were unable to resolve the issue.

Further circumstantial evidence that *Prnp* and *Sinc*/*Prni* were congruent was provided in 1987 by the identification of two amino acid polymorphisms at codons 108 and 189 of the mouse prion protein [45]. It was suggested that these variants could define the two alleles of *Sinc*/*Prni*. $Prnp^a$ (108-Leu, 189-Thr) and $Prnp^b$ (108-Phe, 189-Val) alleles segregated with "short" and "long" incubation time mice respectively. Although widely considered to provide the molecular basis for the segregation of incubation time, this was not conclusively demonstrated until 1998. The advent of gene targeting technology provided the necessary resolution to test the hypothesis that the amino acid sequence of PrP determines incubation time in mouse. Using embryonic stem cells from a $Prnp^a$ (108-Leu, 189-Thr) mouse, Moore and colleagues specifically targeted both codons, changing them to that of

a *Prnp*^{b} allele (108-Phe, 189-Val) [48]. This produced a mouse genetically identical to the original *Prnp*^{a} mouse except at codons 108 and 189 of PrP. Upon challenge with prions, the gene targeted mouse had an incubation time that resembled that of a *Prnp*^{b} allele mouse, thus confirming the central role of PrP amino acid polymorphisms in determining incubation time in mouse.

Based on published genotypes for approximately 60 mouse lines, *Prnp*^{a} is the most common allele with the *Prnp*^{b} only described in 8 strains [46, 49]. This is perhaps not surprising given the high degree of relatedness between laboratory strains of mice [50]. A survey of *Prnp* genotypes from more distantly related mice identified a new allele, *Prnp*^{c} (108-Phe, 189-Thr) [51]. *Prnp*^{c} was identified in MAI/Pas mice derived from *M. m. musculus* wild mice that were trapped in Illmitz, Austria and subsequently inbred at the Pasteur Institute, Paris. MAI/Pas mice have one of the longest incubation times described for the Chandler/RML strain of prion (360 $\pm$ 11); however, at least 100 days of this effect is likely to be the result of other genes, as congenic mice with the *Prnp*^{c} partially isolated on a C57BL/6 background have an incubation time more reminiscent of the *Prnp*^{b} mice (255 $\pm$ 12). These data are supported by the results of comprehensive gene targeting experiments that systematically mutated codon 108 and 189 in turn and produced mice representing each combination of amino acids [52]. It was shown that each codon influences incubation time albeit to different extents. Barron et al. propose that codon 189 has the major effect by controlling the initial interaction and binding to PrP^{Sc} while codon 108 controls the rate of conversion from PrP^{c} to PrP^{Sc} [52].

7 Role of Other Genes

It is clear that *Prnp* is the major genetic determinant of prion disease incubation time; however, it is also apparent that other genes also make a significant contribution to the natural variation observed between different mouse lines. The greatest amount of incubation time data exists for wild type *Prnp*^{a} mice [45, 47, 49, 53, 54]. Within this group, where the PrP coding sequence is identical, the shortest incubation time described for intracerebral inoculation of Chandler/RML prions is 105 $\pm$ 4 in SJL/J mice and the longest is 221 $\pm$ 5 in PWK/Pas mice. This suggests that the combined effect of other genes can extend the incubation time by over 100 days, effectively doubling the incubation time. This is also supported by the congenic studies described above for *Prnp*^{c} on the MAI/Pas and C57BL/6 backgrounds [49]. Indeed, the effect of these genes confounded the interpretation of some of the early studies of the genetic basis of incubation time as it was assumed that they were looking for only one gene.

8 Quantitative Trait Loci Studies

Advances in genetic mapping tools and statistical techniques enabled researches to map regions of the genome harbouring the non-*Prnp* genes controlling incubation time. Using incubation time as a quantitative trait phenotype, microsatellite

markers were used to carry out whole genome screens and linkage analysis provided a statistical measure of the correlation between genotype and phenotype [55–59]. At least five different studies have been published; however, the general approach was similar. In each study, two strains of *Prnp*^{a} mice with different incubation times were used to generate either a backcross or an F2-intercross. All mice were challenged intracerebrally with prions and their incubation times determined. In all crosses, the distribution of incubation times were broad, reflecting the independent segregation of both "short" and "long" incubation time alleles. In simple two way crosses of this nature only one or two recombinations per chromosome can be measured; therefore genotyping was carried out at low resolution with an intermarker distance of 10–20 cM. Together, these independent studies successfully identified multiple loci on eight different mouse chromosomes (Table 1). Because of the low resolution inherent in the study designs, large regions of chromosome, spanning tens of centimorgans, were identified rather than individual genes.

Each of the Quantitative Trait Loci (QTL) studies had unique features that maximised the potential for novel locus discovery but makes comparisons difficult. The first source of variation was the choice of mouse strains. Three different combinations were used: CAST × NZW (F2 intercross) [55, 56], CAST × SJL (F2 intercross) [57] and C57 × RIII (reciprocal backcross and F2 intercross) [57, 59]. Using only two strains of mice in a cross simplifies the analysis but limits the number of potential alleles compared to those expected in an outbred population. The use of closely related laboratory strains also limits the potential for detecting variation. The second source of variation was the choice of prion strain. Across all

Table 1 Summary of published QTL for mouse prion disease incubation time

Cross	Inoculum	Chromosome (cM)	Peak lod score
CAST × NZW	Chandler/RML mouse scrapie	*Mmu2* (61.2)	8.15
F2 intercross[a] ($n = 1009$)		*Mmu11* (43.7)	56.41
		Mmu12 (47.0)	7.55
CAST × SJL	Chandler/RML mouse scrapie	*Mmu9* (17)	5.70
F2 intercross[b] ($n = 153$)		*Mmu11* (43)	5.66
CAST × NZW	Mouse passaged BSE	*Mmu2* (61.2)	5.91
F2 intercross[c] ($n = 124$)		*Mmu11* (43.7)	3.96
F1 × C57 backcross[d]	BSE	*Mmu2* (34)	5.8
($n = 515$)		*Mmu8* (43)	5.2
F1 × RIII backcross[d]		*Mmu4* (42.5)	4.5
($n = 512$)		*Mmu15* (49.6)	3.8
RIII × C57[e] F2 intercross ($n = 282$)	Me7 scrapie	*Mmu5* (68.0)	4.7[f]

[a][55]
[b][57]
[c][56]
[d][58]
[e][59]
[f]Females only

studies, two strains of mouse adapted scrapie (Chandler/RML and Me7) and BSE were used. A unique prion strain is defined by its biochemical properties, pattern of brain pathology and its incubation time; therefore it is to be expected that using different prion strains should identify different QTL [40, 60–62]. A third variable was the use of cattle BSE prions as compared to mouse-adapted prion strains as it is well documented that the presence of a transmission barrier greatly extends the incubation time and QTL identified in this study may be unique to the process of species adaptation.

Although the published QTL studies are difficult to compare, some of the loci may be represented in more than one study, thereby increasing the confidence that a genuine QTL has been found. Two studies using the Chandler/RML prion strain identified multiple loci on chromosome 11 [55, 57]. Given that the peaks of linkage map within 1 cM of each other (*D11Mit36* and *D11Mi219*) and that the confidence intervals are broad, it is reasonable to assume that these crosses have identified the same locus. Both crosses use CAST as one of the mouse strains which may have increased their chances of identifying a common locus. *D11Mit36* is also implicated, although at a suggestive level of linkage, in a smaller CAST × NZW F2 cross inoculated with mouse passaged BSE [56]. These combined findings reinforce the statistical significance of the data and suggest that this locus may act independently of prion strain.

Mmu2 has also been implicated in three studies [55, 56, 58]. The regions identified in the CAST × NZW crosses show considerable overlap, suggesting that this may be the same QTL regardless of prion strain. *Prnp* maps within this region of linkage, making it an excellent candidate gene. However, both CAST and NZW are $Prnp^a$ allele mice and no other amino acid polymorphisms have been described. In addition, no significant differences were detected in PrP expression levels in unaffected mice [55]. Although it is likely that neighbouring genes are the main contributors to this QTL, it remains possible that disease specific differential expression of PrP underlies this QTL.

For all the QTL studies described, it is important to note that it is the effect of the chromosomal region that is detected and not the effect of individual genes. Each locus may represent multiple genes that influence incubation time and the phenotypic effect detected is the net effect of all genes. The ability to detect the effect will depend in part on whether genes in close proximity to each other act in *trans* or in *cis*. Genes acting in *trans* may cancel each other out but genes that act in *cis*, even if their individual effects are small, may amplify the effect over the region. This may confound downstream analysis and verification of candidate genes which may have very small effects on phenotype when analysed individually, in spite of being located in a region of highly significant linkage.

9 From QTL to Candidate Gene

Although very successful, the QTL mapping studies described above identified large genomic regions containing hundreds of genes, which explains why relatively little progress has been made in identifying the underlying quantitative trait genes

(QTG) and polymorphisms. Conventional approaches to fine mapping involve making congenic strains whereby the region of interest is transferred onto the genetic background of another strain by backcrossing with the aim of confirming the QTL by altering the phenotype as predicted by the transferred allele. Although useful in confirming the presence of a QTL, the regions may still be too large for candidate gene analysis.

An alternative approach pursued successfully in our laboratory has been to use a more complex mouse cross to reduce the target region and look at individual candidate genes. In order to fine map a region it is necessary to increase the number of detectable recombinations across the chromosome. This can be achieved using outbred stocks of mice or defined complex crosses [63, 64]. In our case, we have used a heterogeneous stock (HS) of mice which was generated from eight parental strains which were bred semi-randomly over multiple generations [65]. Combined with higher density genotyping, this cross can achieve mapping resolution of approximately 1–3 cM which is a 10- to 20-fold improvement on the original two way crosses [66].

In our studies, 1,000 HS mice at generation 37 were inoculated intracerebrally with the Chandler/RML mouse adapted scrapie strain of prion. To date, we have successfully fine mapped and identified candidate genes on chromosomes 15 and 19 [67, 68]. A 30-Mb QTL on *Mmu15* was previously identified in a C57 × RIII backcross inoculated intracerebrally with BSE [58]. Mapping studies in the HS mice reduced this region to 3.6 Mb with a peak of linkage of $-\log P = 4.52$ [67]. To identify potential QTGs 30 genes were sequenced in the HS parental strains to look for polymorphisms. Comparison of these SNPs with the linkage data and subsequent genotyping of representative SNPs in the HS mice was consistent with *Cpne8* being the most promising candidate gene in the region ($P = 0.0002$). *Cpne8* mRNA was also shown to be upregulated at end stage disease, providing supporting evidence for a role in prion disease. The functional polymorphisms have not been conclusively identified although it is thought that this may be at the regulatory level. *Cpne8* is a member of the copine family which are Ca^{2+} dependent phospholipid binding proteins thought to be involved in membrane trafficking [69]. Although it is not yet clear how Cpne8 influences incubation time, it is possible that it may have some relevance in PrP processing and trafficking within the cell.

The same study design was used to fine map a region of interest on *Mmu19* [68]. In this case a region of 2.9 Mb with a peak of linkage of $-\log P = 5.88$ was identified. After sequencing 22 candidate genes and assessing a number of SNPs, *Hectd2* emerged as the most promising QTG ($P = 0.0008$). As for *Cpne8*, the functional polymorphism was not defined. However, *Hectd2* mRNA was differentially expressed in the presence of different alleles, suggesting that this may be functionally relevant. mRNA was also shown to be overexpressed fivefold at end stage disease as compared to unaffected animals.

Further evidence supporting *Hectd2* as a candidate gene was provided in an association study of HECTD2 SNPs in human prion diseases [68]. From the UK, patients with variant CJD and sporadic CJD were genotyped and compared to a control population. From PNG, kuru patients were genotyped and compared to

elderly women known to have had multiple exposure to kuru but are long term survivors. Initially, a single SNP, *rs12249854* (A/T) was tested and showed that the minor allele (*A*) was significantly over-represented in vCJD ($n = 117$, 8.1%) compared to controls ($n = 601$, 3.9%), $P = 0.0049$, and between sporadic CJD ($n = 452$, 6.3%) and controls, $P = 0.012$. A further seven SNPs in *HECTD2* were analysed. In the UK, strong linkage disequilibrium (LD) was found and an increased risk of vCJD was associated with the haplotype (designated 2) possessing *rs12249854*A ($P = 0.006$). In PNG, however, haplotype 2 showed no significance between kuru and the elderly female survivors of mortuary feasts. Rather, in PNG it was shown that a population specific haplotype (designated 4) was strongly associated with kuru ($P = 0.0009$). Haplotype 4 differs from haplotype 2 at a single SNP, *rs12247672*, which itself is significant in vCJD ($P = 0.0039$) but not at all in kuru ($P = 0.6138$). These data suggest that different functional polymorphisms may be important in the different diseases and populations but that there is consistent evidence of association of genetic variation in *HECTD2* with human prion disease.

As described above, data from both mouse and human studies suggest a role for HECTD2 in prion disease. By homology, HECTD2 is an E3 ubiquitin ligase and, although its substrate is unknown, it is likely to be involved in the ubiquitinylation of proteins for targeted degradation by the proteosome. The ubiquitin-proteosome system has been implicated in prion diseases [70–72] and a number of other neurodegenerative conditions including Alzheimer's (AD) and Parkinson's diseases (PD) [73–75]. These data provide further evidence to support the existence of common pathways and mechanisms in these disorders.

The identification of *Cpne8* and *Hectd2* using HS mice shows the potential for gene discovery using this approach. In addition to fine mapping, the HS mice can be used for assessing the potential of individual candidate genes in association studies. With the caveat that the resolution cannot pinpoint a positive result to a single gene, these studies may provide important additional evidence to support the role of genes originally identified in human studies of prion disease or from other neurodegenerative disorders. As technologies for human genome-wide genotyping have improved, so too have the resources available to mouse geneticists. The availability of a high density mouse SNP genotyping platform now makes a genome-wide screen of the HS mice a realistic possibility. As seen in human studies, these technologies can also provide data on copy number variation which may also be of interest in prion disease.

Future prospects for the discovery of mouse incubation time genes are even better with the advent of a new complex mouse cross as a community resource [76]. The collaborative cross aims to capture the diversity of a heterogeneous stock and the inbred nature of recombinant inbred strains. The starting point for the cross is eight lines of mice chosen for their diversity (A/J, C57BL6/J, 129Sv/Im, NOD/Lt, NZO/HiLt, CAST/Ei, PWK/Ph, WSB/Ei). In the first instance, the mice will be crossed as for a heterogeneous stock to maximise the allelic heterogeneity and then inbred to produce recombinant inbred lines. The aim is to produce ~1,000 inbred lines all of which will be fully genotyped and made available to researchers. Because the genotyping data will already exist, individual researchers need only phenotype the inbred lines. Once linkage has been established, individual candidate

gene analysis will be necessary. It is proposed that each of the mouse lines used to establish the cross will be fully sequenced which will significantly reduce the work load as it will be possible to identify the most promising candidates in silico.

10 Differential Expression

An alternative to identifying genes important in prion disease is to look at differential gene expression. Several small scale studies have identified individual differentially expressed genes such as erythroid differentiation-related factor (EDRF) [77], glial fibrillary acid protein, metallothionein II and B chain alpha-crystallin [78], β-2 microglobulin [79], cathespsin S, C1q β-chain of complement apolipoprotein D and two previously unknown genes named scrapie-responsive gene (ScRG) 1 and 2 [80]. High density microarrays representing approximately 40,000 transcripts in combination with a systems approach has now produced a comprehensive analysis of differential mRNA expression during disease pathogenesis. In the study by Hwang et al., brain gene expression was monitored across eight different mouse-prion strain combinations at regular intervals throughout the incubation time [81]. Subtractive analyses across the experimental groups identified 333 differentially expressed genes. These genes were mapped onto networks and pathways at different stages of disease, thus providing a comprehensive picture of the key cellular events during the disease process. The generation of large networks of genes reflecting the main features of prion disease (PrP^{Sc} replication and accumulation, synaptic degeneration, microglial and astrocyte activation and neuronal cell death) may not highlight key individual genes, such as *Prnp*; however they increase our understanding of the mechanisms of prion disease and may suggest alternative pathways as therapeutic targets.

11 Candidate Gene Approach

The use of mouse models to look at the effects of individual genes on incubation time have proved very useful in confirming the role of proteins that have been implicated in prion disease from a range of different studies. Prion diseases are characterised by the absence of a classical inflammatory response; however, atypical inflammation characterised by microglial activation is central and precedes neuronal death. Consequently, a number of pro- and anti-inflammatory cytokines and chemokines have been investigated.

A straightforward approach to in vivo hypothesis testing is to generate a knockout mouse for the gene of interest, inoculate with prions and look at the effect on incubation time as compared to wild type controls. Experiments of this nature have been carried out for the anti-inflammatory cytokines interleukin-4 (*Il4*), *Il10* and *Il13* and the chemokine, monocyte chemoattractant protein-1 (*Mcp1*) [82, 83]. Following intracerebral inoculation with the RML strain of mouse adapted scrapie, no significant differences were seen for mice deficient in either *Il4*, *Il13* or both. However, for mice

deficient in *Il10*, a highly significant decrease in incubation time was seen from 140 ± 2 (129S1/SvImJ wild type) to 58 ± 14 days [82]. At end stage, these animals had accumulated the same levels of infectivity and neuropathology as wild type mice, suggesting that Il-10 deficiency accelerated the normal disease process. It was suggested that the anti-inflammatory role of Il-10 was usually required to moderate the inflammatory response which, if left unchecked, leads to extensive pathology and death. However this >50% reduction in incubation time contrasts with other data which suggest that $Il10^{-/-}$ mice on a C57BL/6 background show a 19% reduction in incubation time with no difference seen on a 129S1/SvImJ background [84].

Mcp1 was also investigated in this way. The first study showed that, following intracerebral inoculation of the mouse-adapted scrapie prion strain ME7, *Mcp-1* knockout mice a 4 week delay in the onset of late-stage clinical signs as compared to wild type mice [83]. Because *Mcp1* maps to a region of *Mmu11* previously identified as containing a prion disease incubation time QTL, *Mcp-1* was considered a good candidate QTG [85]. Ten polymorphisms were found between the "long" (CAST) and "short" (NZW) incubation time mouse strains, including three amino acid changes. *Mcp1* deficient mice were therefore crossed to both CAST and NZW and challenged with the Chandler/RML mouse scrapie prion strain. In these models, loss of *Mcp1* was not associated with an increase in incubation time, suggesting that the effects of *Mcp1* may be specific to the ME7 prion strain.

Other candidate genes have emerged from a range of other studies. Clusterin (Clu/apolipoprotein J) expression is upregulated in BSE infected mice and has been found to associate with PrP^{Sc} in infected brains [86]. Clusterin has also been implicated in amyloid deposition in Alzheimer's disease and two independent GWAS have shown a significant genetic association between *CLU* and risk of Alzheimer's disease [87]. Following inoculation with BSE prions, *Clu* knockout mice had a 40-day increase in incubation time compared to wild type controls. Pathological examination showed that the absence of clusterin produced less aggregated PrP^{Sc} deposition and more astrocytosis as compared to wild type mice [88].

The most comprehensive study of candidate genes was provided by Tamguney et al. Here they tested 20 candidate genes that were selected on the basis that they had previously been implicated in prion disease or Alzheimer's disease (e.g. *App*) [84]. This included proteins that co-localise or interact with PrP, function in PrP-mediated signalling, glycosylation or protein maintenance (e.g. *Caveolin-1*, *Fyn, Dpl, Hsp70, Sod1*) or are over-expressed in prion disease (e.g. *Il1r, Tgf-β1, Ccr2*). To evaluate their role in prion disease, incubation times were analysed in mouse models where the candidate gene was either mutated, knocked out or over-expressed. Most models showed no significant difference in incubation time as compared to their wild type controls; however, significant differences were seen for the *App* and *Il1r* knockouts and for a transgenic over-expressing human *SOD1*. Incubation times were increased by 13, 16 and 19% respectively. These data illustrate that *App*, *Il1r* and *Sod* may play a central role in the disease process; however, there may not be enough genetic variation between different mouse strains to contribute to the natural variation in incubation time and these genes may therefore not be detectable in QTL studies.

12 MicroRNA

Differential expression studies and candidate gene approaches are useful in identifying genes that may be relevant to the disease process; however it is also important to understand the regulatory processes as these may ultimately be the key to successful therapeutics. MicroRNAs (miRNAs) are short (21–23 nucleotides) single stranded RNAs processed from larger molecules containing stem loops. They are important post-translational regulators of gene expression which are thought to act by complementary base-pair binding to the 3'UTR of mRNA transcripts [89]. Recent advances have implicated miRNAs in several disease processes including prion disease [90]. Saba et al. used microarrays to profile miRNA expression changes in end-stage mouse brains following challenge with the 22A prion strain of mouse adapted scrapie [91]. They showed that 15 miRNAs were differentially expressed including miR-128 that has previously been shown to be upregulated in Alzheimer's disease. The putative target mRNAs of these genes were analysed in conjunction with mRNA expression patterns to refine the profile of prion-related processes particularly for genes involved in key pathways such as protein degradation, cell death signalling and synapse function. Another upregulated miRNA in the Saba et al. study, miR-342-3p, has also been shown to be upregulated in a Macaque model of BSE infection and in human sporadic CJD [92]. This suggests that dysregulation of specific miRNAs may be a common mechanism across different prion diseases.

13 Validation of Candidates

A wealth of genetic studies has now identified multiple candidate genes for prion disease susceptibility, incubation time and disease progression from both human and mouse. For many of these genes the link with prion disease is provided by a statistical association with little functional evidence currently available. The challenge for future work will be to provide compelling functional validation. For many genes, particularly those relating to prion disease incubation time in mice, the gold standard will be to challenge mouse models such as knockouts or transgenics with various prion strains to determine whether incubation time is modified in the predicted way. For other genes, the effects may relate more to PrP^{Sc} deposition, neuropathology and accumulation of infectivity. The systematic analysis of multiple genes in mouse models may be prohibitively expensive and time consuming; therefore alternative approaches are required. One possibility is to take advantage of cell models that are permissive for prion propagation and to combine this with RNAi knockdown technology in a quantitative manner. This may be done either in chronically infected cells or by infecting permissive cells. In the former, the readout indicates whether knocking down expression from the gene of interest results in curing the cells. In the alternative model, a measure of cell susceptibility, following candidate gene knockdown, is provided by counting the number of infected cells present at the end of the assay. These cellular systems cannot model all aspects of prion biology; however, they may prove a useful tool in guiding future experiments.

14 Conclusion

In both human and mouse, mutations and polymorphisms in the prion protein play a major role in determining disease susceptibility. However, it is now clear from a large number of studies that the prion protein alone does not account for the natural variation seen in susceptibility and incubation time and several other proteins must play a role in the disease process. It is the polymorphisms in these genes and their regulators that explain the remaining variation observed in the population. The identification of these genes and their associated alleles will be key to improving patient diagnosis and genetic counselling and is expected to inform epidemiological modelling of the consequences of population-wide exposure to BSE prions where the number of silently infected individuals from primary or secondary (iatrogenic) exposure remains unclear. The identification of new genes and pathways will also increase our understanding of what constitutes susceptibility and incubation time and provide new avenues for therapeutic intervention.

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Atypical Prion Diseases in Humans and Animals

Michael A. Tranulis, Sylvie L. Benestad, Thierry Baron, and Hans Kretzschmar

Abstract Although prion diseases, such as Creutzfeldt–Jakob disease (CJD) in humans and scrapie in sheep, have long been recognized, our understanding of their epidemiology and pathogenesis is still in its early stages. Progress is hampered by the lengthy incubation periods and the lack of effective ways of monitoring and characterizing these agents. Protease-resistant conformers of the prion protein (PrP), known as the "scrapie form" (PrP^{Sc}), are used as disease markers, and for taxonomic purposes, in correlation with clinical, pathological, and genetic data. In humans, prion diseases can arise sporadically (sCJD) or genetically (gCJD and others), caused by mutations in the PrP-gene (*PRNP*), or as a foodborne infection, with the agent of bovine spongiform encephalopathy (BSE) causing variant CJD (vCJD). Person-to-person spread of human prion disease has only been known to occur following cannibalism (kuru disease in Papua New Guinea) or through medical or surgical treatment (iatrogenic CJD, iCJD). In contrast, scrapie in small ruminants and chronic wasting disease (CWD) in cervids behave as infectious diseases within these species. Recently, however, so-called atypical forms of prion diseases have been discovered in sheep (atypical/Nor98 scrapie) and in cattle, BSE-H and BSE-L. These maladies resemble sporadic or genetic human prion diseases and might be their animal equivalents. This hypothesis also raises the significant public health question of possible epidemiological links between these diseases and their counterparts in humans.

M.A. Tranulis (✉)
Norwegian School of Veterinary Science, Oslo, Norway
e-mail: Michael.Tranulis@nvh.no

S.L. Benestad
Norwegian Veterinary Institute, Oslo, Norway

T. Baron
Agence Nationale de Sécurité Sanitaire, ANSES, Lyon, France

H. Kretzschmar
Ludwig–Maximilians University of Munich, Munich, Germany

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Contents

1 Introduction

Certain brain diseases of humans and animals known as *proteinopathies* are associated with the accumulation of misfolded proteins both inside and around neurons [1]. Alzheimer's disease in humans is by far the most common member of this group. The prion diseases, exemplified by Creutzfeldt–Jakob disease (CJD) in man, scrapie in small ruminants, and bovine spongiform encephalopathy (BSE) in cattle, constitute a peculiar sub-group within proteinopathies by being experimentally transmissible [2]. Because of this feature, together with the sponge-like appearance of vacuoles in affected brain areas, prion diseases are also known as *Transmissible Spongiform Encephalopathies (TSEs)*.

Although, the exact nature of transmissible prions is still unresolved, the single essential component seems to be an abnormal isoform (PrP^{Sc}) of the cellular prion protein (PrP^{C}) [2–5]. Somehow, already misfolded PrP, possibly as protofibrils or oligomers, recruits normal PrP^{C} and orchestrates a templated misfolding of its substrate with considerable precision, allowing a step-by-step growth of the less soluble and partly proteinase-resistant PrP^{Sc} protein structure. Thus, during prion propagation, there is a flow of structural information encoded in protein scaffolds. According to this scheme of protein "inheritance," subtle differences in the three-dimensional arrangement of misfolded PrP (derived from different species etc.) are maintained, allowing for a certain spectrum of misfolded conformers. This phenomenon probably accounts for most of the variability between prion isolates [6]. A prion isolate is a preparation from diseased brain, capable of transmitting prion disease in a suitable host, without regard to the detailed physical nature of the pathogen.

Experimental transmission of prion isolates has allowed them to be characterized in terms of incubation periods [7], as well as by clinical pathology and histopathological appearance, which are manifest as spongiform change and reactive gliosis [8]. A brain pool from three scrapie-diseased sheep (SSBP/1) revealed, upon transmission in goats, two distinct clinicopathological syndromes, namely a "scratching" variant and a "drowsy" variant [9], thereby providing the introduction to the field of prion strain research. Passage of the drowsy goat strain in mouse resulted in the isolation of the mouse-adapted "Chandler" strain [10], from which the RML (Rocky Mountain Laboratories) strain was also derived. Further passage

of the drowsy variant into two lines of mice, expressing different PrP alleles, resulted in isolation of the 79A and 87V strains [11], while another mouse-adapted scrapie strain, which was derived from a field case, was named ME7 [11]. When this strain was propagated in hamsters, disease occurred only after a very prolonged incubation period and the strain was termed ME7-H [12]. However, if the ME7 strain was passaged once in transgenic mice expressing a mouse/Syrian hamster chimeric PrP (SHa/Mo PrP) and then back into hamster, a short incubation period and an altered disease profile was observed. This demonstrated that the characteristics of a prion strain can be influenced by its passage history [13]. Due to the obvious problem of generating a multitude of different prion strains in laboratory rodents, and that of limited relevance to real-life situations, strain variation between prion isolates nowadays refer to isolates derived from identical PrP sequences (e.g., sheep with identical PrP genotypes) which, upon serial passage in congenic hosts, result in distinct disease phenotypes with respect to incubation periods and lesion profiles [14].

In striking parallel to the drowsy and scratchy variants observed in goats, a similar division into drowsy (DY) and hyper (HY) phenotypes was also observed following inoculation of Syrian hamsters with brain material (Stetsonville isolate) isolated from mink that had succumbed to transmissible mink encephalopathy (TME), a rare, foodborne prion disease of unknown origin [15, 16]. Studies of the DY and HY strains led to the seminal discovery that there were distinct differences in the biochemical properties of PrP^{Sc} proteolytic fragments from the two variants. The DY derived PrP^{Sc} was also shown to be more sensitive to proteolytic degradation. Since these differences could not be explained by variation in incoming or host PrP primary sequences, it was proposed that the strain characteristics could be encrypted in the three-dimensional arrangement of the PrP^{Sc} structures [17], without the aid of pathogen-specific nucleic acids, as previously suggested [18]. Further evidence in favor of the protein-only hypothesis was gained when similar strain-related PrP^{Sc} types were propagated in a cell-free system [19]. With the development of the protein misfolding cyclic amplification assay (PMCA) [20], various studies of the cell-free propagation of PrP aggregates and their strain characteristics have been conducted. These have included the de novo generation of infectivity from recombinant PrP substrates in the presence of accessory cellular components [21], and very recently, even without any mammalian co-factors [4]. These have provided further compelling evidence in support of the protein-only hypothesis, but do not exclude important roles for accessory components in prion propagation.

Several investigations have substantiated the use of biochemical PrP^{Sc} properties as reliable strain-correlated signatures, particularly for distinguishing variant CJD (vCJD) from other forms of CJD [22, 23], but also in comparison of sporadic CJD and genetic prion disease in humans [24, 25]. Importantly, the relative abundance of different glycoforms of the proteinase K (PK)-resistant PrP-fragments, the so-called glycoform profile, was also taken into consideration. Based upon these principles, two major classification systems for human prion isolates were developed [22, 23, 26–29], of which the system proposed by Parchi and co-workers [23, 30] has been most widely used. In this system, two principal PrP^{Sc} types were recognized:

Type 1, with an apparent molecular mass of 21 kDa (lower band), and Type 2, with a corresponding band of 19 kDa. In concert with the polymorphic variation at codon 129 (methionine/valine, M/V) in the *PRNP* gene, this described a total of six subtypes of sCJD. However, the types MM1 and MV1 were merged as indistinguishable, while MM2 cases included two variants, based on histopathological changes predominantly in the cerebral cortex (MM2-Cortical) or thalamus (MM2-Thalamic) [31]. It was soon necessary to expand this relatively simple classification system when it was discovered that Type 1 and Type 2 could frequently co-occur in patients [32–35], increasing the total number of pure and mixed subtypes to 12 [36]. Interestingly, the relative ratio of Type 1 and Type 2 varied between brain regions in patients with mixed subtypes, indicating nuances in the cellular tropism of the varieties. Despite the complexity of PrP^{Sc} typing in CJD, an inter-laboratory analysis involving seven diagnostic laboratories reported excellent agreement in assessment of cases [37]. Thus, full validation of these methods seems to be within reach. Co-occurrence of strains has also been observed in transgenic mice expressing the human PrP gene ("humanized" mice), inoculated with vCJD isolates. Surprisingly, some of the mice propagated a strain similar to sCJD in the brain, while simultaneously propagating the vCJD strain in the spleen [38]. Co-occurrence of classical and atypical/Nor98 scrapie in sheep has also been reported [39].

In addition to strain-related variation in the size and glycosylation of PK-resistant PrP^{Sc} fragments, the denaturation kinetics of misfolded PrPs in the presence of chaotropic salts, as measured by increased binding of conformational antibodies (epitope exposure), substantiated the link between misfolded conformers and strains [40, 41]. These studies also revealed that a significant proportion of misfolded PrP^{Sc} could be protease-sensitive in a strain-specific manner, thus highlighting the potential weakness of PK-based methods for detection of misfolded PrP^{Sc}, at least for some strains. However, as greater PK-sensitivity often correlates with less stable PrP-aggregates, this attribute can be utilized for diagnostic and classification purposes by combining a conformation stability assay [41, 42], in which PrP-aggregates are adsorbed to membranes before exposure to denaturants [43], with Western Blots, allowing mapping of N-terminal cleavage end-points. This has been useful in discriminatory epitope-mapping of many prion strains, not least BSE and atypical/Nor98 scrapie [44–48].

By combining these methods for PrP^{Sc} typing with neuropathological lesion profiles [49] and, importantly, patterns of brain PrP^{Sc} deposits [50–53], prion strains can be fairly well characterized. However, current protocols are quite cumbersome and time-consuming. Therefore, other approaches for discriminating between prion strains, using conformation-sensitive probes [54] or cell culture systems [55], have been advocated.

Despite steady progress in non-bioassay discrimination of prion strains, the mouse bioassays remain superior. This was recently demonstrated when two strains of chronic wasting disease (CWD), a prion disease affecting cervids, with biochemically indistinguishable PrP^{Sc} aggregates, were recognized as separate CWD-1 and

CWD-2 strains [56], following transmission to specific lines of transgenic mice expressing cervid PrP [57].

Prion diseases are traditionally grouped according to their route of acquisition, as sporadic, familial, or infectious diseases. While sporadic disease variants predominate in humans, no such prion disease has yet been formally identified in animals.

One explanation that has been proposed for the differences in occurrence of human and animal prion diseases is age-distribution. The farm animal population at highest risk for development of sporadic or familial prion disease is the elderly segment, which is normally quite small in production animals. The likelihood of discovery is further reduced by low disease incidence and challenging diagnostics. Thus, large-scale surveillance programs, with particular scrutiny of the populations at risk, have proved vital in the discovery of atypical ruminant prion diseases, as detailed in this chapter.

Foodborne prion disease in humans was discovered in Britain in 1996 and resulted in a public health crisis of unprecedented proportions. Strong circumstantial evidence [58–61] pointed to BSE as the cause of the disease, which was termed variant CJD (vCJD) [62]. The clinicopathological features of vCJD clearly differed from those of other human prion diseases, but were similar to those seen in transmissions of BSE in cattle [59, 63]. The molecular signature of the agent of BSE has been remarkably preserved across species barriers. The glycoprofile of the BSE agent is characterized by a predominance of diglycosylated forms. In order to avoid confusion with atypical forms of BSE, the original BSE isolate will hereafter be named classical BSE (BSE-C). The two recently discovered forms of atypical BSE [64, 65] are referred to as H-type BSE and L-type BSE, because the unglycosylated band of PrP^{Sc} is higher (H-type) or lower (L-type) than that of BSE-C. The L-type BSE strain is probably identical to the originally described bovine amyloidotic spongiform encephalopathy (BASE). One fundamental difference between BSE-C and the atypical forms is the age of disease onset in affected cattle; whilst for BSE-C the age of disease onset is typically in the 3–5 years range, for H-type and L-type BSE the average age of disease onset is over 8 years.

Classical scrapie in small ruminants, the archetypal animal prion disease [66], and CWD in cervids are the only known prion diseases that spread effectively within a species, under natural (free-ranging wild population) or near-natural (extensive husbandry) conditions [67–70]. Spread may be horizontal through direct contact or shared environments [71, 72], or vertical from mother to offspring during the pre- or neonatal period [73–75]. Although these diseases clearly illustrate that prion diseases can behave as true infectious maladies, they could, ironically, turn out to be exceptions to the rule rather than typical. Predators have the potential to be exposed to ruminant prion disease and, as evident with the BSE-C agent, possibly develop prion disease. A recent study of predators in a CWD endemic area of Wisconsin did not provide evidence of a transmission of CWD prions across species barriers [76]. For a prion disease to be sustained as infectious within a population, it seems that the infectious prion must be present at relatively high levels in the peripheral tissues of affected animals, most notably in the digestive

tract and its associated salivary glands and lymphoid aggregates. CWD transmits effectively, with saliva [71, 72, 77], feces [78], and urine [79] as primary vehicles, either through contact or via the environment. Protease-resistant PrP of cervid origin has been detected in water samples taken from a CWD endemic area [80].

Traditionally, transmission of classical scrapie has been considered to occur primarily from mother to offspring in the perinatal period, possibly related to the placenta, amniotic fluid, and fetal membranes that are discarded at parturition [73]. Contamination of lambing areas is reported to increase the exposure risk, but transmission via pastures [81] is probably underestimated. PrP^{Sc} has been detected in gut-associated lymphoid tissues in natural and experimental scrapie [27], not only in the ileum and jejunum, but also in rectal tonsils [82], indicating that infectious agents are probably shed in the feces. Recently, PrP^{Sc} has also been detected, after amplification in PMCA, in swabs taken from the oral cavities of sheep harboring classical scrapie [83], again providing evidence that gastrointestinal tract secretions probably have a role in the horizontal spread of classical scrapie. Transmission through milk and colostrum might also occur [75]. Consequently, it is very difficult to establish effective control measures against the spread of classical scrapie through management adjustments at the flock-level. Normally, PrP-genetics, which strongly modulate the peripheral prion distribution and classical scrapie incidence, must be taken into account [84].

With the discovery of ruminant prion diseases that resemble sporadic human prion disease, a certain element of "balance" in the epidemiology of prion disease could be established. However, the logic of this could also be reversed, and an important question could be raised: might the low level occurrence of spontaneous farm animal prion disease underlie one or more forms of human prion disease, which are currently misinterpreted as spontaneous? This major epidemiological and public-health issue will be the focus of discussion in this chapter.

2 Atypical Human Prion Disease

Human prion diseases, which can be (1) sporadic and of unknown origin, (2) acquired by transmission from humans or animals, or (3) of genetic origin caused by mutations of the PrP gene (*PRNP*), display a large number of pathological and clinical changes. These include small or large vacuoles, either generalized or restricted to certain areas, synaptic, perivacuolar, plaque-like PrP deposits, kuru plaques, multicentric plaques, and florid plaques. Most cases show a rapid cognitive decline and myoclonus, while some display ataxia or visual signs or sleep disturbances. For a long time the variety of changes was seen as a continuum, with only a few, mainly clinical, subtypes recognized. These included the Heidenhain variant of CJD with predominant myoclonic and visual symptoms, the Brownell–Oppenheimer or ataxic variant, or Gerstmann–Sträussler–Scheinker (GSS) as a hereditary ataxia with cognitive decline and multicentric plaques. In order to be able to assess "atypical" prion diseases in humans, one first needs to consider the large variety

of "typical" disease phenotypes, i.e., those that form well-defined subgroups (types) from a pathological, clinical or biochemical point of view, and that occur with a certain, and often predictable, regularity. Genetic prion diseases, i.e., GSS, gCJD, and fatal familial insomnia (FFI), consist of a number of heterogeneous subgroups and are associated with various *PRNP* mutations and large phenotypic variability that have been described in great detail and are outside of the scope of this review.

With the advent of molecular characterization of human prion diseases, well-defined subtypes of sporadic and acquired CJD have become apparent. First, it was shown that the two genetic PrP variants encoding either methionine (M) or valine (V) at codon 129 not only define susceptibility to sporadic and acquired CJD, but also determine the pathology [85–87]. Subsequently, it was realized that PK-resistant PrP in humans can be observed in two different forms (PrP^{Sc} Type 1 and 2) distinguishable by two preferential PK digestion sites at amino acids 96 and 85 that also affect the pathological and clinical appearance of the disease [23]. This has resulted in the recognition of six types of sporadic CJD, that have been well characterized with regard to their molecular composition, i.e., genotype at codon 129 (MM, MV, or VV) and PrP^{Sc} Type 1 or 2 and pathological and clinical phenotype in the following ways [31, 88]. (1) The MM/MV1 type, in its pure form, comprises 40% of all sCJD cases, it shows small vacuoles, synaptic PrP^{Sc} deposition, and clinically is characterized by rapid cognitive decline, typical periodic sharp wave complexes (PSWC), and positive 14-3-3. (2) The VV2 type (15% of sCJD cases) shows plaque-like deposits and perineural staining in the cortex as well as plaque-like deposits in the cerebellar cortex and white matter. Clinically gait ataxia is prominent in early stages. (3) The MV2K type is found in 8% of all sCJD patients and shows kuru plaques on histological examination. Clinically, dementia and ataxia are characteristic features. (4) The MM2 C type is very rare in its pure form (1% of all sCJD cases), and its hallmark is widespread, large, confluent vacuoles in the cerebral cortex with perivacuolar and coarse focal PrP^{Sc} staining. (5) The MM2T type is also very rare (about 1%), and is indistinguishable from FFI, which is caused by a D178N mutation in coupling with 129 M. Clinically, insomnia is prominent, while pathologically there is atrophy of the thalamus and the inferior olive, with only minor changes in other regions of the brain. (6) The VV1 type is also very rare (1%) and affects young patients (average age 39 years). Pathologically, there is severe spongiform change in cortico-striatal regions, but only very faint punctate PrP^{Sc} staining on IHC.

In addition to the above six pure phenotypes, it has become apparent that up to 30% of sCJD cases can be "mixed," with co-occurrence of PrP^{Sc} types and parallel pathological phenotypes [36, 89]. Type *MM/MV1 + 2C* is the commonest mixed type (43% of all MM cases), and pathologically shows large confluent vacuoles in some areas in addition to the small vacuolar change typical of MM/MV1. Type *VV2 + 1* is only recognized when considering the Western blots, as the histological appearance is very similar to the pure VV2 type. In contrast, types *MV 2K + 2C* and *MM2T + 2C* can only be diagnosed on histological grounds (both have PrP^{Sc} Type 2 pattern in Western blots).

Acquired human prion diseases include vCJD, iCJD, and kuru. While individual cases of iCJD and kuru may be indistinguishable from sCJD, vCJD, the only human prion disease known to be transmitted from a non-human source, is clearly distinct from a pathological, clinical, and biochemical view [62]. The fully-fledged phenotype, with abundant pathology, including florid plaques in the CNS, has only been described in codon 129 MM-homozygous patients. Two MV heterozygous individuals showed PrP^{Sc} deposits in the spleen after transmission of the disease by blood transfusion or batches of the blood clotting factor VIII, but no CJD-related brain pathology [90, 91]. Experiments in transgenic mice expressing human PrP suggest that different pathological phenotypes should be expected in codon 129 MV or VV individuals if they develop CJD after extended incubation periods [92, 93].

Kuru, a human prion disease formerly transmitted by ritual cannibalism in New Guinea, is histologically indistinguishable from sCJD in individual cases; however, it has been noted that kuru shows considerable similarities to sCJD VV2 or sCJD MV 2K [29, 94–97].

Using archival tissues from transmission experiments, Parchi and co-workers recently provided evidence that strengthened the theory that kuru originated from cannibalism of an individual with sCJD VV2 or MV 2K, since no indication of MM1 prions could be found, even in subjects carrying the MM or MV genotype and that had succumbed to kuru [98].

The total number of iCJD cases was reported to have reached more than 400 in 2006 [99]. Of the almost 200 dura mater implant-associated iCJD cases more than 50% (132 cases) have been identified in Japan. Whether transmitted by treatment with contaminated human growth hormones, dura mater implants, or other carriers, iCJD has been described as histologically indistinguishable from sCJD. However, some cases transmitted by dura mater implants are notable exceptions. These patients show slow progression of disease, an absence or late occurrence of sharp wave complexes on EEG and, most intriguingly, PrP-positive plaques in the brain, some of which are surrounded by vacuoles and are reminiscent of florid plaques. In a recent survey, Yamada and co-workers found that in Japan the plaque-type accounted for 52% (14 of 27 cases) of the pathologically diagnosed iCJD cases after dura mater implants; all of these cases were codon 129 homozygous for methionine [100]. Two similar cases have also been noted outside of Japan [101, 102]. The Western blot in the few cases investigated have shown peculiarities, with a deviating glycoform ratio in one case [102] and an unglycosylated band of intermediate size between Type 1 and Type 2 in almost all cases [100, 102]. Based on the results of transmission experiments to transgenic mice, Kobayashi and colleagues have suggested that plaque-type iCJD may be caused by transmission of sporadic CJD-VV2 or MV2 prions to persons with a codon 129 MM genotype [103]. It appears that iCJD that is transmitted through contaminated growth hormone also shows PrP-positive kuru plaques in affected individuals of all three possible codon 129 constellations, including MM [99].

In addition to these types, i.e., the six types of sCJD, the two types of iCJD, and – so far – the single type of vCJD, there are some cases that cannot be grouped according to the above scheme. Such cases are considered “atypical,” and they can be divided into two groups.

The first group, described by Gambetti and co-workers in 2008, represents a novel human disease with protease K-sensitive PrP, for which they coined the term "protease-sensitive prionopathy" or PSPr [104]. This disease was first described in 11 codon 129 valine-homozygous patients, who demonstrated cognitive decline and behavioral changes. In six of ten patients with obtainable pedigrees there were family histories of dementia, but no *PRNP* mutations were found. There was an unusual pattern of pathological changes, consisting of minimal spongiform degeneration with vacuoles larger than those found in typical CJD and a peculiar immunohistochemistry (IHC) with PrP^{Sc}-positive dots (microplaques) and large granules in the cerebellum and cortex not seen in typical CJD. Typical PK-resistant PrP was undetectable with standard diagnostic procedures. Abnormal PrP was detectable only after enrichment, and at much lower concentrations, than in typical CJD and formed a distinct ladder-like electrophoretic profile. The total abnormal PrP concentration was described as 16 times less, and the PK-resistant portion, as 4 times less than in sCJD MM1. Western blots with monoclonal antibody (mAb) 1E4 showed a ladder of PK-resistant fragments ranging from 29 to 6 kDa, mostly in subcortical regions and mostly undetectable by mAb 3F4. An approximately 6-kDa unglycosylated band was found that is reminiscent of a 7-kDa band that occurs in GSS. This disease type may not be particularly rare; in the US National Surveillance Center, it was found in 3% of all sCJD cases and in 16% of all valine-homozygous CJD cases. Subsequently, similar cases were encountered in the UK [105] and the Netherlands [106]. One case was arguably identified by German Surveillance before the term PSPr was coined [107]. Because it now seems that all three codon 129 phenotypes (MM, MV, and VV) can be afflicted and that protease resistance may vary, the term "variably protease-sensitive prionopathy (VPSPr)" has been suggested for this disease [108]. The transmissibility of VPSPr is still unresolved. Atypical/Nor98 scrapie shares some of the pathological and biochemical features of VPSPr, with a relatively PK sensitive PrP^{Sc} [109], giving rise to several bands in Western blots including low molecular mass bands and a tendency to form plaque-like or dot-like deposits in immunohistochemistry.

The second group consists of mostly single cases of CJD with unusual and inexplicable changes, which do not fit with the classification system described above. These include one CJD MM1 case with kuru-plaques [110], three CJD MM1 cases of long duration and PrP-positive plaques in the white matter [111], one MM case in which PrP^{Sc} lacked the doubly glycosylated band [112], one case with widespread PrP deposition and an unusual PrP^{Sc} type [113], and various others [114]. Considering the thousands of CJD cases that have been investigated in recent years, these cases must be considered as exceptionally rare. The etiology and pathogenesis of these cases remain unclear, and genetic factors and the possibility of an infectious origin must be considered.

The above combinations of molecular determinants and phenotypic sets are considered typical subgroups, or even strains, of disease that have been confirmed. Transmission studies of human cases are complicated by the observation that after inter-species transmission, the size and glycoform of the human PrP^{Sc} inoculum may change [115, 116]. In a recent review of transmission studies in non-human

primates performed at the National Institute of Health and summing up the results of other groups, Parchi et al. [98] were able to show four different strains of the most common CJD types and kuru. However, whether the rare sCJD MM 2C and VV1 cases as well as PSPr/VPSPr are additional strains awaits clarification.

3 Atypical/Nor98 Scrapie

During a period of enhanced awareness regarding sheep scrapie in the late 1990s, a few suspect scrapie cases, with unusual clinicopathological features, were identified in Norway, and were subsequently named Nor98 [45]. The first case appeared in Eastern Norway, until then considered to be a scrapie-free region. Intriguingly, old and healthy sheep in the same flock were found to be carrying PrP-genotypes conferring high susceptibility to scrapie, while the affected animal had a PrP genotype rarely seen in classical scrapie [117]. The predominant clinical symptom was progressive ataxia. While in classical scrapie the predominance of pathology is in the brain stem, the unusual Nor98 cases showed pathology and PrP^{Sc} accumulation in the cerebral and cerebellar cortices. The glycoprofile of proteinase-resistant PrP^{Sc} from all the unusual cases also differed from classical scrapie, with multiband pattern and a characteristic lower band of around 11 kDa, not previously described in animal prion diseases (Fig. 1). In the brain stem, pathology and PrP^{Sc} accumulation was mild or even below detection limits [45]. This lesion profile has proved to be consistent for atypical/Nor98 scrapie [118–121]. Furthermore, when PrP^{Sc} is detectable in the brain stem in atypical cases, it is mainly restricted to the neuropil of the spinal tract nucleus of the trigeminal nerve, as sparse and diffuse

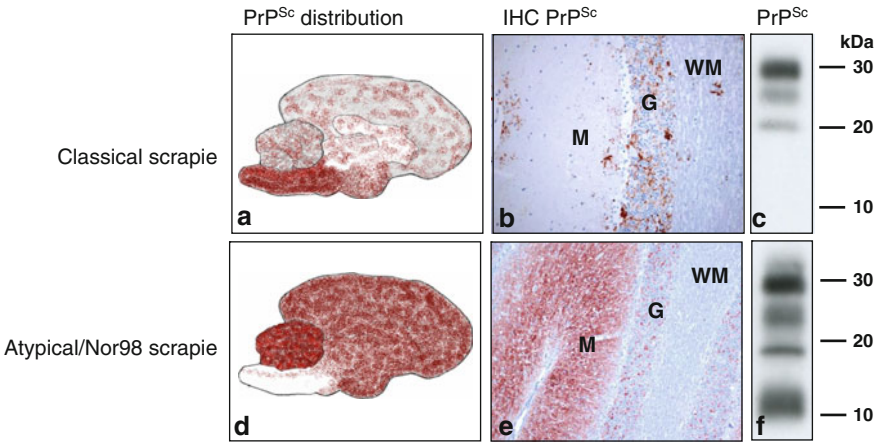

Fig. 1 Comparison of classical scrapie and atypical/Nor98 scrapie with regard to overall distribution of PrP^{Sc} in the brain (drawings **a**,**d**), immunohistochemical PrP^{Sc} staining patterns in the cerebellum (**b**,**e**) and PrP^{Sc} banding pattern as revealed in Western Blots (**c**,**f**)

staining, and/or as globular staining in the white matter, which is occasionally pronounced along the spinocerebellar tracts.

In addition to the difference in lesion profile, the pattern of PrP^{Sc} deposits in such atypical scrapie cases also differs from that seen in classical scrapie cases [109, 122]. In classical scrapie, a stellate pattern of PrP^{Sc} deposits is particularly prominent in the granular layer of the cerebellum, while in atypical/Nor98 scrapie a widespread, diffuse, synaptic type of staining can be observed in the molecular or the granular cell layers or both, and may be variably associated with globular staining in the white matter.

In the cerebral cortex, classical cases display a stellate, perivascular, and frequently intraneuronal PrP^{Sc} staining, while in atypical/Nor98 cases a diffuse laminar staining dominates. In some atypical/Nor98 cases, multifocal dense plaque-like aggregates can be seen in the white matter tracts [118].

One case of atypical/Nor98 scrapie has been reported in which all brain sections appeared negative for immunohistochemical PrP^{Sc}; however, a faint staining could be observed in the granular layer by use of the PET-blot technique [109, 122]. Interestingly, there appears to be no correlation between the degree of PrP^{Sc} staining and the disease phenotype, regardless of age and PrP genotype.

Since the obex area and especially the dorsal motoric nucleus of the vagus nerve (DMNV) were previously considered the tissue of choice for scrapie diagnostic purposes, the Nor98 cases might easily have escaped identification. At around the same time that the first atypical/Nor98 cases were discovered, a number of atypical scrapie cases were identified through pan-European screening programs, designed particularly for detecting BSE in small ruminants. Following a report of German and French cases [46], further reports came from many other European countries [120, 123–129], as well as The Falkland Islands [130], USA [131], New Zealand [132], Australia [133], and, most recently, Canada [134]. According to data from the European Surveillance Program for TSE in small ruminants, atypical/Nor98 scrapie has been identified in more than 20 European countries [135], with a surprisingly uniform prevalence (about 6–8 cases per 10 000 tests), which contrasts with the more variable and clustered occurrence of classical scrapie. A study of formalin-fixed archival brain material has back-dated atypical/Nor98 scrapie in the UK to at least 1987 [136], demonstrating that atypical/Nor98 scrapie has existed in the small ruminant population for years without being detected.

Diagnostic kits for rapid detection of atypical/Nor98 scrapie have recently been evaluated by the European Food Safety Authority (EFSA), with five out of nine tests being approved [137]. Since tests that did not achieve EFSA approval, due to lack of sensitivity, will be in use until the end of 2010, it is reasonable to assume that atypical/Nor98 scrapie is underdiagnosed. The underlying problem of detecting atypical/Nor98 scrapie seems not only related to the selection of tissue for analysis, but also the relative PK-sensitivity of PrP^{Sc} generated in this disease [48, 119, 138], a property that continues to be preserved following transmission into mice. In the first study of transmission of atypical/Nor98 scrapie to transgenic mice expressing ovine PrP, similar lesion profiles, PrP^{Sc} deposition patterns, incubation periods, and PrP-glycoprofiles were reported following inoculation from isolates obtained from

three Norwegian and ten French atypical scrapie cases, suggesting that a single, unique prion strain was responsible for causing the atypical/Nor98 type of scrapie [139]. This study has been extended to British atypical isolates, and the results again confirm the uniformity in transmission characteristics of atypical/Nor98 isolates [140].

Detailed mapping of the PrP-fragments, as revealed in Western Blots after PK treatment, has demonstrated, despite some small discrepancies, a unique banding pattern, with a characteristic lower band (band C after PNGase-F treatment) of about 11 kDa [44, 45, 48] or 7 kDa [138], consisting of an internal PrP-fragment. The N-terminus of the 11-kDa fragment has been accurately mapped to amino acid (aa) 85–90, since several mAbs with known linear epitopes are available for this region of the PrP. The C-terminus of the fragment is predicted to be around aa 185–190, assuming no post-translational modifications. However, there are only few mAb available to the aa 155–200 region in PrP, and thus precise epitope mapping of the C-terminus of this PrP-fragment is currently problematic. The 11-kDa band is a consistent diagnostic hallmark in cases of atypical/Nor98 scrapie, not only in sheep, but also in goats [141].

The occurrence of classical scrapie in sheep is modulated by three polymorphisms, at codons 136 (alanine/valine, A/V), 154 (arginine/histidine, R/H), and 171 (glutamate/arginine/histidine, Q/R/H) in the gene that encodes PrP, *PRNP* [142–144]. The frequency of these, and other PrP polymorphisms, varies significantly between breeds and the different polymorphisms are generally mutually exclusive. The valine polymorphism at codon 136 results in the major disease promoting allele $V_{136}R_{154}Q_{171}$ (VRQ), with homozygous VRQ/VRQ animals the most susceptible. The presence of arginine (R) at codon 171 (ARR allele) has been shown to be significantly associated with a reduction in the probability of classical scrapie occurrence, conveying a relative resistance, thereby enabling marker-assisted breeding for resistance towards classical scrapie [145, 146].

However, atypical/Nor98 scrapie seems to occur most frequently in sheep with PrP genotypes considered to be less susceptible, while apparently sparing flock-mates carrying high susceptibility PrP genotypes [45]. A large proportion of atypical/Nor98 cases carry at least one AHQ allele. Moreover, the presence of phenylalanine (F) at codon 141 (wt-allele leucine, L), giving rise to the $AF_{141}RQ$ (AFRQ) allele, is also strongly associated with this type of scrapie [147–149]. A schematic presentation of allelic modulation of susceptibility for classical and atypical/Nor98 scrapie is given in Fig. 2. Interestingly, atypical/Nor98 scrapie has been very rarely recorded in sheep carrying the VRQ allele, and only in combination with a disease promoting AHQ or AFRQ allele, while several cases have been seen in sheep with the ARR/ARR genotype [150]. In a study of genetic risk for atypical/Nor98 scrapie involving 248 cases [151], the most susceptible PrP genotypes were found to be ALHQ/ALHQ, ALHQ/AFRQ, and AFRQ/AFRQ, while heterozygotes ALHQ/ALRQ and AFRQ/ALRQ were less susceptible. This result concurs with data from other studies [44, 148, 150] and also highlights the previously reported [150] risk of atypical/Nor98 scrapie in animals with the ALRR/ALRR genotype, which are enriched in marker-assisted breeding.

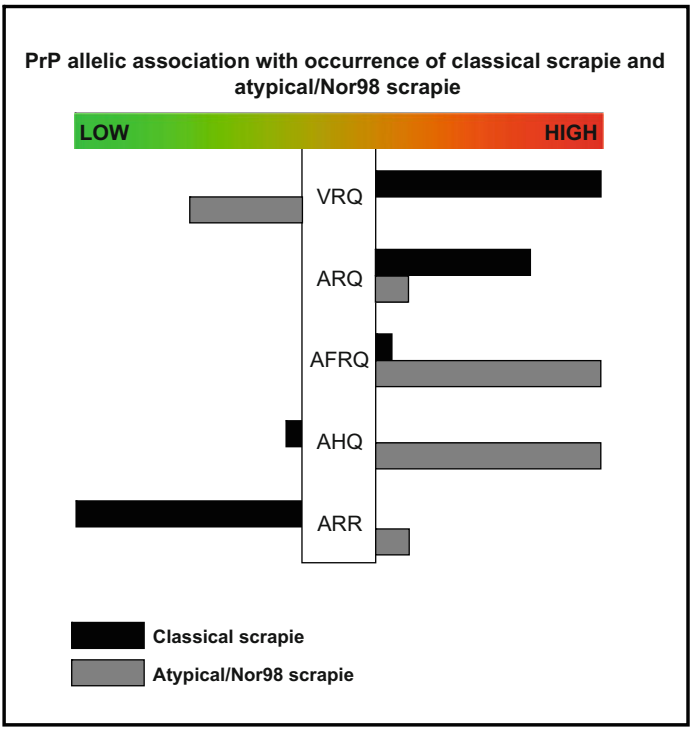

Fig. 2 Cartoon of PrP allelic association with occurrence of classical scrapie and atypical/Nor98 scrapie. While classical scrapie occurs most frequently in sheep with VRQ and ARQ alleles, atypical/Nor98 scrapie occurs most frequently in AFRQ and AHQ PrP alleles. The presence of the ARR allele confers strong resistance towards classical scrapie, while this is not the case for atypical/Nor98 scrapie

Although some data indicate a protective effect of the VRQ allele towards atypical/Nor98 scrapie, the first transmission to transgenic mice (line tg338) was achieved with mice carrying the ovine PrP VRQ/VRQ genotype on a PrP null background. However, these mice over-expressed PrP by eight- to tenfold, compared to the level in sheep brain [139]. In a subsequent study, aimed at defining the transmission characteristics in more detail, three lines of TgOvPrP4 mice were used, with neuron-specific expression of the ovine ARQ/ARQ genotype at approximately 0.25 times, 1.5 times, and 6 times the level in sheep. In this study, transmission of atypical/Nor98 scrapie was shown to be more strongly modulated by host PrP expression level and the genotype of the inoculum than is observed with classical scrapie or BSE-C [152].

Epidemiological analyses of atypical/Nor98 scrapie in different countries [135, 150, 153, 154] have resulted in a fairly uniform epidemiological profile being described. Atypical/Nor98 scrapie is widely distributed geographically, without clustering, and normally only one or very few cases are seen, even in flocks of several hundred animals. Additionally, atypical/Nor98 cases are generally older

than classical scrapie cases and a significant proportion of the cases have been identified through screening-programs and/or by analysis of fallen stock, while this is uncommon for classical scrapie [118]. Case-control studies [154, 155] of atypical/Nor98 scrapie have failed to identify epidemiological evidence to indicate an infectious behavior, whereas contact and purchase are important risk factors for classical scrapie [67]. However, epidemiological analysis and modeling of sheep prion disease is far from trivial. For a comprehensive review of this topic, see [156].

In 1980, Gibbs and co-workers [157] transmitted kuru, CJD, and scrapie to squirrel monkeys by the oral route. The scrapie isolate they used had a complex passage history, with nine serial passages from sheep to goat, followed by three serial passages in mice, and finally three serial passages in hamster. Two monkeys were fed brain, kidney, and spleen from scrapie-infected hamsters for 3 days. The monkeys succumbed to spongiform encephalopathy after 25 and 32 months. Notably, the incubation period in a squirrel monkey intracerebrally inoculated with brain material from a scrapie-diseased goat was 31 months and that of intracerebral inoculation of mouse-adapted scrapie isolates varied from 14 to 31 months. Thus the incubation periods observed after oral inoculation were comparatively short, indicating that the oral route of transmission could be effective, at least in combination with large doses of infective material. This significant study demonstrated that a rodent-adapted scrapie strain can be transmitted to non-human primates via the oral route and also provides a pertinent reminder of the pathogenic potential of scrapie. Transmissions of classical and atypical/Nor98 scrapie to mice have produced disease profiles and PrP^{Sc} types that are reassuringly different from those seen in BSE and in human prion disease [59, 139, 152, 158]. However, transmission of a primary classical scrapie isolate from Romanov sheep to C57BL/6 mice produced a lesion profile and PrP^{Sc} type conspicuously similar to those seen after transmission of sCJD and iCJD to the same line of mice, while this was not the case after transmission of a mouse-adapted scrapie strain included in the same study [158]. Thus, despite the lack of recognized epidemiological connections between sheep scrapie and human prion disease [159], and considering the variability in strain characteristic of sheep scrapie, it should be noted that we currently have no effective experimental models for estimation of human risk related to oral exposure to such pathogens.

4 Atypical BSE

Until 2004 it was believed that BSE was a unique disease, due to a single major strain of TSE agent that was propagated in cattle by recycling in contaminated meat-and-bone meal. This opinion had been strongly supported by the identification of a unique strain of transmissible agent, as defined by its features following transmission of the disease in inbred wt mouse models that had been extensively used in the UK to demonstrate that scrapie in sheep and goats could involve a variety of different strains [160, 161]. Transmission to mouse lines, such as RIII,

C57BL, or VM, of different bovine isolates, including those from different countries, revealed a uniform behavior, as shown by incubation periods of the disease, distribution of spongiform changes, and the distribution and features of PrP^{Sc} deposits. This uniformity of the disease in cattle was also supported by the findings of the same distribution of lesions in the brains of cattle, with the medulla oblongata predominantly involved, including at different times of the epidemic in cattle in the UK, or in other European countries [162–164].

In 2004, this established "fact" that BSE was a unique single strain infection, was abruptly challenged when two countries reported the occurrence of a few cases of BSE in older cattle that showed deviant features of the disease. In France, three cases were reported that showed an unusually high molecular mass of PrP^{Sc} by Western blot studies, indicating a protease cleavage site distinct from that seen in cattle with "classical" BSE (BSE-C) [64]. This was unlikely to be a reflection of phenotypic variations since a number of studies had previously shown that the PrP^{Sc} molecular features, notably the protease cleavage site, was a very reliable characteristic of BSE-C, even preserved upon transmission to other species, such as in variant CJD in human [22], or, experimentally, in sheep or in mice [28, 165–168]. A second report from Italy described two cases from cattle, but in these cases the PrP^{Sc} molecular mass was slightly lower than that observed in BSE-C, and, more strikingly, the proportion of the diglycosylated band was significantly lower [65]. In this report, the differences from classical BSE were further supported by histopathological analysis that revealed that the distribution and nature of lesions in the cattle brains differed distinctly from those previously described during the BSE-C epizooty. Not only were the lesions much more abundant in the cortical regions of the brain, in contrast with the preferential location in the brain stem in BSE-C, but were also characterized by the presence of amyloid plaques which are not observed in BSE-C. Due to this last characteristic, the disease was termed Bovine Amyloidotic Spongiform Encephalopathy (BASE). As histopathological data are not always available, the two diseases identified in France and Italy were soon referred to as either H-type BSE or L-type BSE, based on the differences in PrP^{Sc} molecular masses identified after Western blot analysis [169]. Importantly, in all five cases, analysis of the coding sequence of the *PRNP* gene did not reveal any unusual features compared with the known *PRNP* sequence in cattle, and thus a genetic origin of such cases seemed unlikely.

These unexpected observations prompted further investigations, initially directed towards the confirmation of the original findings, including in other countries. Other "atypical" cases of BSE in cattle were rapidly identified in a number of different countries in Europe (Germany, Netherlands, Poland, Denmark, Ireland, United Kingdom, Sweden, Austria) [167, 168, 170–173] and also in Japan [161], USA [174, 175], and Canada [176] (Table 1). Questions immediately arose about the possible origins of such cases. Interestingly, some of the cases had been diagnosed in countries considered at low risk of exposure to contaminated meat-and-bone meal such as Sweden in Europe, or the USA. The findings were also confirmed in Italy, where two additional BASE cases (BSE-L) were recognized and, to a larger extent, in France where the highest number of "atypical" cases have

Table 1 Atypical BSE cases recognized globally

Country	H-type	L-type
Germany	1	1
Austria	–	1
Denmark	–	1
France	14	13
Ireland	1	–
Italy	–	3
Netherlands	1	2
Poland	2	8
United Kingdom	3	–
Sweden	1	–
Switzerland	1	–
Canada	–	1
USA	1	–
Japan	2	1
SUM	27	31

Table updated as of August 2010

been identified to date since the implementation of active surveillance by rapid tests in 2000 (a total of 27 cases, 14 H-type and 13 L-type). Indeed, all cases recognized so far have been identified after initial diagnosis by rapid tests through the active surveillance programs that encompass exhaustive surveillance in adult cattle in Europe since 2001. This large-scale screening has certainly been essential for the discovery of such cases, which hardly would have been identified by passive surveillance, with the only exception being the finding of a single H-type BSE case in a 17-year-old zebu kept in a zoological park in Switzerland, in which clinical signs triggered neurological investigations, including BSE testing [177].

All the available studies to date have demonstrated that the cases identified in different countries have molecular features that are extremely consistent with those of the very first cases diagnosed in France and Italy. Among these studies, a collaborative European network study of atypical cases from six European countries (France, Italy, Netherlands, Germany, Poland, and Sweden) confirmed the migration properties and glycosylation profiles (glycoprofiles) of the H- and L-type isolates (Fig. 3). The presence of H-type BSE could also be detected by the differential binding of mAb specific for N- and more C-terminal PrP regions, indicative of the existence of two PrP^{Sc} populations differing by their N-terminal cleavage sites [178] (Fig. 3). In addition, this study revealed that both H-type and L-type BSE have enhanced protease sensitivities at pH 8, compared with BSE-C isolates. These properties appear to be consistent within each BSE type, independent of geographical origin, and although not all these criteria have been investigated in all the studies, this was also confirmed in cases of H-type BSE reported in the UK, USA, and Canada, and for cases of L-type BSE diagnosed in Japan and Canada. Thus, altogether these studies provided reliable criteria for identifying two atypical BSE forms, in addition to classical BSE.

Importantly, diagnosis of BSE in cattle is not always followed by further analyses to identify whether the case is classical or atypical. This contrasts with

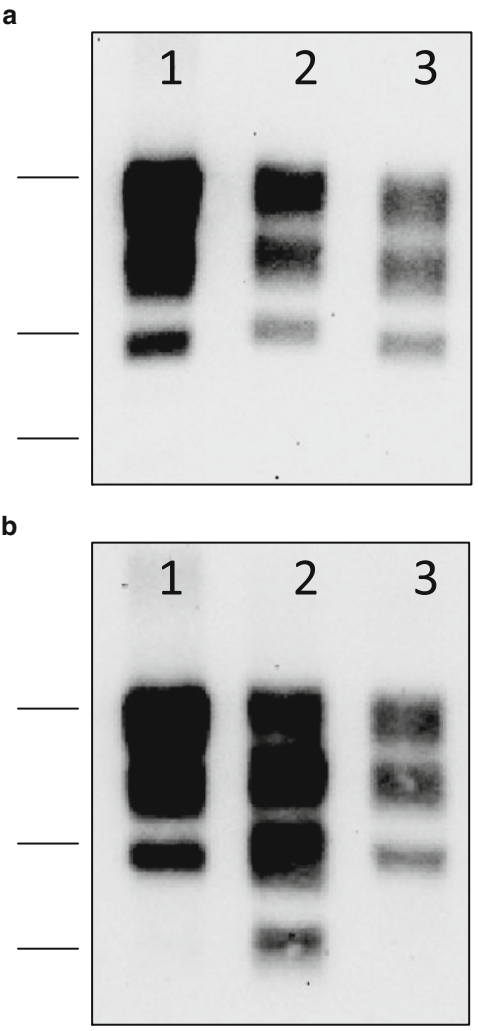

Fig. 3 Western Blot analysis of PrP^{Sc} in classical BSE (lane 1), H-type BSE (lane 2) and L-type BSE (lane 3). (**a**) Detected with mAb Sha31 (epitope aa 156–163). (**b**) Detected with mAb SAF84 (epitope aa 175–180). In (**b**) an additional band at approximately 14 kDa is detected and distinct proportions of the PrP^{Sc} bands were detected for H-type BSE (lane 2). Size markers in kilodaltons (29.0, 20.1, and 14.3) are indicated on the *left*

the diagnosis of prion disease in sheep and goats in Europe, following which further molecular investigations are undertaken on a regulatory basis, in order to identify possible unusual features that could indicate infection by the BSE agent. This difference in approach suggests that the number of atypical BSE cases in cattle among those reported by the OIE, as shown in Table 1, is certainly underestimated. The largest published study was performed on cattle in France between 2001 and 2007, during which 13 atypical cases (7 H-type and 6 L-type) were identified among 645 BSE cases [179]. During the 6-year period, 17.1 million rapid tests were performed, including 3.6 million animals over 8 years old. Thus, the estimated frequencies of H-type and L-type BSE were 0.41 and 0.35 per million adult cattle

tested, respectively (1.9 and 1.7 in cattle over 8 years old). The most important information obtained in this study was the striking difference between the distribution by year of birth of atypical and classical cases detected during 2001–2007. Whereas the cases with classical BSE clearly indicated exposure to the classical BSE agent during 1993–1996 in France, one or two cases born each year were eventually identified as having atypical BSE (both H-type and L-type) at a mean age of 12 years. Overall, whereas the frequency of such atypical cases, although possibly underestimated, is certainly low, another relatively large series of atypical BSE cases (mostly of L-type) was also reported in Poland in cattle over 9 years old [172]. Together with the finding of atypical cases in some countries that were considered at very low risk of foodborne classical BSE, these data strongly argue that such atypical BSE cases represent sporadic TSE, as are most cases of CJD in humans. The *PRNP* gene has not been characterized for all cattle diagnosed with atypical BSE. However, genetic analyses have so far failed to identify any polymorphisms specifically associated with atypical BSE in the gene that encodes PrP [64, 65, 161, 164, 169, 175, 177]. Interestingly, a single case was reported in the USA with H-type Western blot features, which also had an E211K mutation, similar to the mutation E200K in humans, the most frequent mutation associated with gCJD [174]. Although this finding is of interest regarding our basic knowledge about the possible relationship between the PrP sequence and the pathogenesis of such diseases, it is unlikely to be of particular significance in cattle populations, since it seems that this mutation is quite rare, as it was not detected in a study of 6,062 cattle in the USA [180]. As well as the *PRNP* coding sequence, one or two copies of a distinct *PRNP* haplotype were identified in five of six atypical BSE cases from France, Canada, and USA [170, 181]. This haplotype spans a portion of *PRNP* that includes part of intron 2, the entire coding region of exon 3, and part of the three prime untranslated region of exon 3 (13 kb), suggesting that a genetic determinant in, or near, the *PRNP* gene may influence susceptibility of cattle to atypical BSE. Also, whereas two regulatory region polymorphisms in the PrP gene of cattle have been reported to be associated with resistance to BSE, it has also been shown that the promoter polymorphism correlation was specific to classical BSE, and that atypical BSE or experimentally inoculated TSE bypass the site of influence of the polymorphisms [182]. This suggests that genetic features involved in classical or atypical BSE could be linked to the different pathogeneses of the diseases, putatively in relation to either their acquired or sporadic origins.

Another line of investigation that followed the initial discovery of the two atypical forms of BSE was related to the potential transmissibility of the agent from such cases, and biological features of the TSE agents involved. Transmission of H-type BSE through a species barrier was first demonstrated using C57Bl/6 wild-type mice [183]. Detailed studies using transgenic mice expressing bovine or ovine PrP eventually demonstrated that H-type BSE and L-type BSE exhibit different biological and molecular features in these mouse models, and that both also differed from the well-known strain isolated from classical BSE [169, 184–188]. In particular, the distinct properties of PrP^{Sc} in cattle identified by Western blot were maintained following transmission to transgenic mice expressing bovine PrP, in association with distinct incubation periods of the disease, as well as distribution and features of PrP^{Sc} deposits

in the mouse brains [169, 184–188]. Transmission of H-type BSE into C57Bl/6 wild-type mice demonstrated the presence of a second, more C-terminally cleaved, form of PrP^{Sc} (PrPres #2), which, in unglycosylated form, migrated at approximately 14 kDa and could be specifically detected using C-terminal antibodies, as in the cattle brain [189]. Overall, these data clearly indicate that the three phenotypes of BSE in cattle are associated with three different major strains of transmissible agents.

To date, few studies have been published with regard to the transmission of these atypical BSE forms in cattle. Intra-species transmission of an Italian case of L-type BSE between Friesian and Alpine brown cattle was demonstrated using intra-cerebral inoculation, and the L-type molecular features were reproduced, as well as the presence and distribution pattern of amyloid plaques in the cattle brains [190]. This was associated with a disease phenotype characterized by mental dullness and progressive amyotrophy, suggestive of a motor neuron disorder, in contrast with the over-reactivity and hypersensitivity in the absence of muscle changes, which are observed in cattle infected by a classical BSE isolate [190]. Transmission between cattle by the intra-cerebral route was also reported from other L-type isolates identified in Japan [191] and Germany [197] also from H-type cases from the Netherlands or Germany [192, 197] that also confirmed that the H-type molecular features were maintained in experimentally infected cattle. However, the extent to which these two atypical forms of BSE could also be transmitted to cattle by the oral route is currently unknown, and this information is essential in order to estimate more precisely the potential risks associated with these rare BSE isolates.

However, experimental studies in mouse models have already generated the hypothesis that such putatively sporadic cases may have been the origin of the foodborne epizooty of classical BSE, possibly after first recycling through an intermediate species [184, 188, 198]. Inoculations of C57Bl/6 and SJL wild-type mice with an Italian L-type BSE isolate resulted in some intriguing observations [188]. Unlike with inoculation with a classical BSE isolate, in these inoculations no evidence of transmission was detected during the first passage; clinical disease and brain lesions were absent and PrP^{Sc} could not be detected either by Western blots or immunohistochemistry. However, a second passage from a pool of brain homogenates from these mice, and inoculated into the same mouse lines by both intra-peritoneal and intra-cerebral routes, resulted in clinical disease associated with brain lesions. Surprisingly, the molecular features, as well as the distribution of spongiform changes and PrP^{Sc} deposits, showed considerable similarities with those found in the same mouse lines when infected with classical BSE. It was thus assumed that either (1) classical BSE was present as a minor component in the initial L-type BSE inoculum and had been propagated in these mice or that (2) in the mouse background the L-type BSE had converted into classical BSE. More recently, the emergence of a strain indistinguishable from classical BSE has also been described following serial passages of BSE-H in some C57Bl/6 mice, although BSE-H can also be transmitted and maintained with the characteristic H-type properties in this mouse model [189, 198]. Another study of L-type BSE transmitted to a transgenic mouse line (tg338) over-expressing the ovine PrP (VRQ allele), also showed a shift in disease phenotype, with molecular and lesional features similar to those observed in these mice following transmission of classical BSE [184]. These results

suggest the theoretical possibility that an early event, preceding the amplification of the classical BSE agent in cattle, could have been recycling of a TSE agent primarily present in cattle but modified through passage in an intermediate host such as sheep. However, it remains to be demonstrated whether these two series of observations reflect the genuine identification of a common TSE agent present in both L-type and classical BSE in cattle, or if this represents phenotypic convergence following transmission in mouse models. Full characterization of the biological properties of the TSE agents produced in these experiments will help to clarify this issue.

Although, the characteristics of transmission of L-type BSE in sheep still remain to be described, it should be noted that different results were obtained in another transgenic mouse line (TgOvPrP4) expressing the ARQ allele of the ovine PrP at a lower level and under a different promoter [193]. In this mouse line, the molecular and lesional features of L-type BSE remained clearly distinct from those observed following transmission of classical BSE [193]. Results from this study had similarities to those obtained following transmission of the Stetsonville TME isolate in the same mouse model, having first been transmitted to cattle. This has been further supported by a recent study showing the transmissibility in Syrian hamsters of both L-type BSE and TME-in-cattle, and their similar molecular features in this model, as well as in bovine transgenic mice [199]. It can thus be hypothesized that outbreaks of TME in minks, which are occasionally observed in captive minks as a foodborne disease of unknown origin, might actually be caused by the consumption of food derived from cattle affected with L-type BSE. If confirmed, this would demonstrate the oral transmissibility of this atypical BSE form in a non-ruminant species. This hypothesis appears to have gained further credence from the recent observation of phenotypic similarities between TME in cattle and L-type BSE transmitted to macaque monkeys [194]. This recent study [194] belongs to a series of investigations that were initiated to evaluate the potential for transmission of atypical BSE to humans using experimental studies in non-human primates and in transgenic mice expressing the human PrP. This question was first raised following the initial discovery of BASE in Italy, which revealed certain molecular and lesional similarities to a sub-type of sporadic CJD (M/V Type 2) [65]. In another study, some molecular similarities were also emphasized regarding the identification of comparable C-terminally cleaved PrP^{Sc} products in both H-type BSE in cattle and in some CJD cases in humans [189]. This was recently reinforced by the observations that the amounts of the 13-kDa C-terminal fragment were specifically increased in CJD Type 1 cases, but this fragment was virtually undetectable in variant CJD and in most sporadic CJD subtypes associated with Type 2 (except the MM2 thalamic sub-type) [195]. While H-type BSE and L-type BSE are already strongly reminiscent of molecular Types 1 and 2, respectively, in sporadic CJD, these observations rather suggest that both cattle and humans could be affected by diseases involving similar molecular mechanisms. However, given the enigmatic origin of such diseases, it is obviously important to investigate any possible causal link between these human and bovine diseases.

In this context, successful transmission of L-type BSE has been reported in a cynomolgus macaque monkey inoculated by the intra-cerebral route. This infection

was characterized by a shorter survival period (26 months with 25 mg cattle brain inoculated) than in monkeys infected with classical BSE (40 months with 100 mg cattle brain inoculated), and distinctive clinical, neuropathological, and biochemical features were recorded [194]. Molecular similarities with a rare MM2 cortical subtype of sCJD in humans were also observed, rather than with the MV2 subtype. Transmission of L-type BSE has also been demonstrated by the intra-cerebral route in a transgenic mouse line expressing normal levels of the human M129 PrP protein, and a higher (60%) transmission rate than that observed with classical BSE was reported [196]. Similar conclusions were reached from another study using another transgenic mouse line that over-expresses (about sixfold) M129 human PrP [186]. This study also concluded that there is no significant species barrier for L-type BSE between cattle and humans, as an attack rate of 100% was observed, and the incubation time was not reduced upon secondary passage. In contrast, transmission of the classical BSE agent to the same mice showed a substantial species barrier effect.

5 Conclusions

As elaborated in the preceding sections, various questions still need to be resolved, for an accurate assessment of the public health risk associated with the steadily growing variety of prion diseases that are identified as occurring in farmed ruminants. Accurate estimates of prevalence of prion disease in farm animals and analysis of transmission properties of the corresponding agents are the cornerstones of such assessments, and this means that costly and extensive surveillance programs deserve to be continued. In addition, the transmission properties of these diseases must be scrutinized by transmission studies in transgenic mice expressing human PrP (different PrP genotypes), as well as further transmissions in primates.

Despite the obvious need for accruing more data, our current knowledge indicates that it is highly unlikely that prion infected farm-animal derived foods or products are a significant source of human prion diseases.

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Chronic Wasting Disease

Sabine Gilch, Nandini Chitoor, Yuzuru Taguchi, Melissa Stuart, Jean E. Jewell, and Hermann M. Schätzl

Abstract Chronic wasting disease (CWD) is a prion disease of free-ranging and farmed ungulates (deer, elk, and moose) in North America and South Korea. First described by the late E.S. Williams and colleagues in northern Colorado and southern Wyoming in the 1970s, CWD has increased tremendously both in numerical and geographical distribution, reaching prevalence rates as high as 50% in free-ranging and >90% in captive deer herds in certain areas of USA and Canada. CWD is certainly the most contagious prion infection, with significant horizontal transmission of infectious prions by, e.g., urine, feces, and saliva. Dissemination and persistence of infectivity in the environment combined with the appearance in wild-living and migrating animals make CWD presently uncontrollable, and pose extreme challenges to wild-life disease management. Whereas CWD is extremely transmissible among cervids, its *trans*-species transmission seems to be restricted, although the possible involvement of rodent and carnivore species in environmental transmission has not been fully evaluated. Whether or not CWD has zoonotic potential as had Bovine spongiform encephalopathy (BSE) has yet to be answered. Of note, variant Creutzfeldt–Jakob disease (vCJD) was only detected because clinical presentation and age of patients were significantly different from classical CJD. Along with further understanding of the molecular biology and pathology of CWD, its transmissibility and species restrictions and development of methods for preclinical diagnosis and intervention will be crucial for effective containment of this highly contagious prion disease.

Keywords Cervid prions · Chronic wasting disease · Prion containment · Wild-life prion disease · Zoonotic potential

S. Gilch, N. Chitoor, Y. Taguchi, M. Stuart, J.E. Jewell, and H.M. Schätzl (✉)
Department of Veterinary Sciences, University of Wyoming, 1174 Snowy Range Road, Laramie, WY 82070, USA
and
Department of Molecular Biology, University of Wyoming, 1000 E. University Ave., Laramie, WY 82071, USA
e-mail: hschatzl@uwyo.edu

Contents

1 Introduction and History

Chronic wasting disease (CWD) is unique amongst prion diseases as it affects both wild-living and farmed animals, while it resembles scrapie in sheep in tissue distribution, horizontal spread, and environmental implications. CWD is a prion disease of mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*), Rocky Mountain elk (*Cervus elaphus nelsoni*), and less frequently of moose (*Alces alces shirasi*) [1–3]. Presently it occurs only in parts of North America (USA and Canada), although it was exported from Canada to South Korea [4–7]. CWD as a disease entity was first described in the 1970s by the late E.S. Williams (Department of Veterinary Sciences, University of Wyoming) and colleagues in captive mule deer and elk [2, 3] in northeastern Colorado and southeastern Wyoming, and was correctly classified by these authors as a prion disease. Initially considered a rare and exotic disease, CWD has emerged in recent years as probably the most puzzling and certainly the most contagious member of the prion diseases. From the putative epicenter in Colorado and Wyoming where CWD is highly endemic now, it has spread north to Canada (Alberta and Saskatchewan), south to New Mexico, and east to New York and West Virginia. Remarkably, there are unaffected states in between and the overall distribution appears discontinuous. Therefore, it is difficult to discern whether CWD was endemic there previously or whether increased awareness and improved diagnostics are explanations. From careful retrospective and prospective surveillance and diagnosis studies in certain Wyoming hunting areas, it appears more likely that CWD historically and presently is expanding in both prevalence rate and geographic distribution (Todd Cornish, personal communication).

Outside of North America, CWD has only been found in South Korea, where it came via import from Canada [4]. European countries, including Germany and

Belgium, have reported limited surveillance data, mainly in the predominant species roe deer (*Capreolus capreolus*) [8, 9]. Some member states of the European Union are conducting targeted surveillance programs in wild and farmed red deer, as experimental transmission of CWD to European red deer (*Cervus elaphus elaphus*) was demonstrated [10].

Control or containment of CWD is presently not possible as non-domestic animals are affected whose migration is hard to restrict. In addition, wildlife cannot be subject to the usual anti-prion culling strategies used with domestic herds. Although depopulation is done in affected captive herds, targeted elimination by sharpshooters of clinical suspect or at-risk animals in the wild proved to be ineffective. The obvious impact of persistence in the environment on transmission further complicates the situation, as does the lack of methods for preclinical diagnosis. This review discusses current research in CWD with the focus on genetics and co-factors of susceptibility, intra- and inter-species transmission, existence of strains, and zoonotic potential.

2 Epidemiology

CWD was first observed as a disorder in captive mule deer at the Colorado Division of Wildlife Foothills Wildlife Research facility in Fort Collins, Colorado in the late 1960s. Original assumptions were that the disorder was the result of nutritional deficiency and/or stress reflective of captivity. The disease was not identified as a prion disease until the late 1970s [2]. Since its identification, CWD has been discovered in 17 states of the USA and 2 Canadian provinces. CWD has also appeared in a farm at Chungbuk, South Korea after importation from Canada (Table 1; [4] *Global Invasive Species Database;* http://www.issg.org/database).

First identification of CWD occurred in captive mule deer of Colorado, followed by identification in captive mule and black-tailed deer in a research facility in Wyoming in 1979 and the diagnosis of a captive elk that same year. In 1981 the Colorado Division of Wildlife identified CWD in a wild elk, the first diagnosis of a wild cervid. By 1985, the Colorado Division of Wildlife confirmed CWD in a wild mule deer and the Wyoming Game and Fish Department identified the disease in the first wild cervid of the state, also a mule deer. An “endemic zone” was established for CWD spanning Northern Colorado and South Eastern Wyoming, but by 1996 a captive elk farm in Saskatchewan tested positive for CWD. The first state to identify CWD in a wild population before its diagnosis in captive animals was Nebraska in 1999 (see Fig. 1).

While several initial state cases have been identified in captive herds, there is no epidemiological evidence that can point to an origin for CWD. Numerous hypotheses have been suggested including mutation/modification of scrapie strains to affect cervids, or spontaneous conversion of normal cervid PrP into PrP^{Sc} that is transmissible to other elk and deer. No clear cut origin for the disease may be discovered. Extensive surveillance and modeling programs have been developed

Table 1 Summary of chronic wasting disease discoveries throughout the world (modified from www.cwd-info.org *CWD timeline*)

Year	Species	Status	Location
1967	Mule deer (not identified as prion disease)	Captive	Colorado
1979	Mule deer	Captive	Wyoming
	Black-tailed deer	Captive	Wyoming
	Elk	Captive	Wyoming
1981	Elk	Wild	Colorado
1985	Mule deer	Wild	Colorado
	Mule deer	Wild	Wyoming
1996	Elk	Captive	Saskatchewan
1997	Elk	Captive	South Dakota
1999	Mule deer	Wild	Nebraska
2000	Mule deer	Wild	Saskatchewan
2001	White-tailed deer	Wild	South Dakota
	White-tailed deer	Captive	Nebraska
	Elk	Captive	Korea
2002	White-tailed deer	Wild	Wisconsin
	Mule deer	Wild	New Mexico
	Elk	Captive	Minnesota
	White-tailed deer	Captive	Wisconsin
	Elk	Captive	Oklahoma
	White-tailed deer	Wild	Illinois
	Elk	Wild	South Dakota
	White-tailed deer	Captive	Alberta
2003	Mule deer	Wild	Utah
2005	White-tailed deer	Captive	New York
	White-tailed deer	Wild	New York
	White-tailed deer	Wild	West Virginia
	Moose	Wild	Colorado
	Mule deer	Wild	Alberta
	Elk	Wild	New Mexico
2006	White-tailed deer	Wild	Kansas
	White-tailed deer	Captive	Minnesota
2008	Elk	Wild	Saskatchewan
	White-tailed deer	Captive	Michigan
	Moose	Wild	Wyoming
2010	White-tailed deer	Wild	Virginia
	White-tailed deer	Captive	Missouri
	Mule deer	Wild	North Dakota

for CWD in a number of locations. Based on this research, prevalence in captive herds can reach greater than 90%, and in free ranging herds as high as almost 50% ([11]; Wyoming hunting area 65; http://gf.state.wy.us/downloads/pdf/CWD%20Deer%20Map.pdf).

While susceptibility to CWD appears uniform between sexes in captive deer, in free ranging mule deer populations prevalence is higher among mature males [12]. Also it has been observed that recruitment is affected by CWD but current research implies that the results may not significantly impact population growth rates [13].

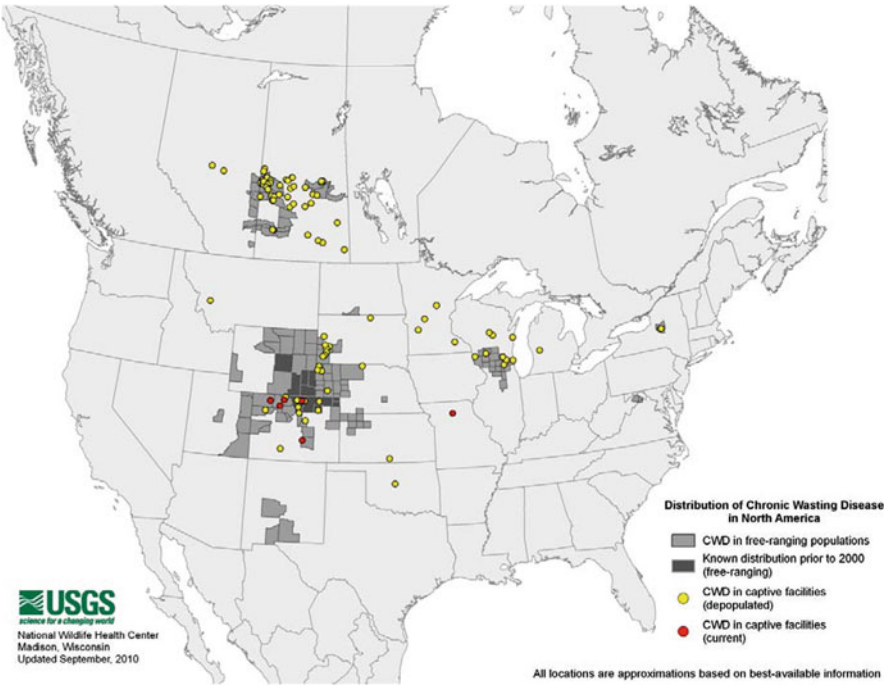

Fig. 1 Distribution of CWD in North America. *Gray shading* indicates CWD in free-ranging populations, *dark gray* as known so prior to 2000. *Yellow circles* denote CWD in captive facilities which were depopulated, *red circles* current facilities. Data taken from USGS and National Wildlife Health Center Madison, Wisconsin

3 Clinical Signs and Pathology

3.1 Clinical Pathology

Description of the clinical symptoms of CWD mainly comes from observation of captive or experimentally infected animals. Prognostic symptoms are absent during early and mid-phase of disease. Body mass changes can be seasonal and some behavioral changes fall into the normal repertoire of cervids. Leading clinical symptoms are progressive weight loss (Fig. 2a) and pronounced behavioral changes like depression and isolation from herd, although during the terminal stage hyperexcitability can also manifest. Other symptoms can be hypersalivation/sialorrhea (excess of saliva either due to increased salivation or difficulty in swallowing), odontoprisis (teeth grinding), ataxia with head tremor, esophageal dilation and regurgitation, aspiration pneumonia, and polyuria/polydipsia. Terminally, animals may walk repetitively around the perimeter of their enclosure or have a fixed stare with no awareness of their surroundings [11]. The duration of clinical disease is very variable. "Sudden death" in captive animals is rare. A slowly progressive

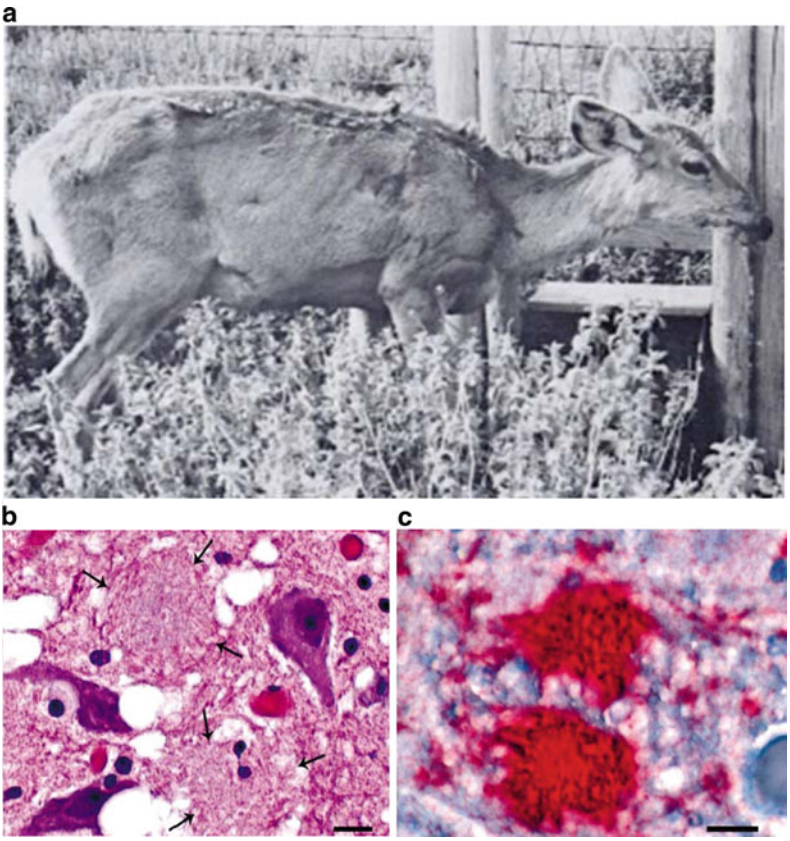

Fig. 2 Clinical aspect and brain pathology. *Upper picture* shows a female mule deer, 3 years of age, with clinical signs of late chronic wasting disease, which include hypersalivation, emaciation and depression. *Lower pictures* depict amyloid plaques in the thalamus of a WTD with CWD. *Left* shows formalin-fixed, paraffin-embedded tissue stained with HE. Plaques are delineated with *arrows*. *Right picture* shows an immunohistochemical (IHC) staining of such amyloid plaques. Pictures kindly provided by the abatement of the late Beth Williams, University of Wyoming

clinical course is seen for some weeks or months and death typically occurs within 4 months, though a few animals survive up to a year [14]. In free-ranging animals the period may be shorter due to compromised ability to forage or find water. The animal can also be an easy target for predators. Environmental stressors such as extreme cold bring about death of affected animals even under captive conditions.

3.2 Histopathology

The histopathology of CWD affected animals is largely consistent with that seen in other animal prion diseases [15–17]. Lesions are observed in the gray matter of the

central nervous system (CNS). The nature or severity of the lesions does not change in brains of natural or experimental cases (of intracerebral or oral route exposure). Lesions are bilaterally symmetrical and anatomically constant among animals. Spongiform appearance is obvious; vacuolization occurs in neuronal perikarya and neuronal processes. Along with neuronal degeneration, astrocytic hyperplasia and hypertrophy may appear. Hematoxylin–eosin (HE) staining of brains of affected animals reveals amyloid plaques (Fig. 2b). Plaques appear as pale fibrillar, eosinophilic areas of neuropil and are sometimes surrounded by vacuoles (florid plaques) [16–18]. Detection of PrP^{Sc} in brain sections by immunohistochemistry (IHC) provides a very good means of visualizing CWD pathology, while maintaining the structural context (Fig. 2c). A drawback of IHC is that it is not useful in diagnosis of sub-clinical CWD as spongiform encephalopathy occurs around the same time clinical features develop [11]. PrP^{Sc} is detected by IHC or immunoblot in a variety of tissues in cervids showing clinical symptoms [15, 19]. Though PrP^{Sc} can be found in regions of the brain not exhibiting spongiform change, typically there is correlation between PrP^{Sc} deposition and spongiform appearance [15].

3.3 Pattern of PrP^{Sc} Accumulation

Usually, the nucleus of the vagus nerve is most severely affected [14, 20]. Accumulations of PrP^{Sc} have been observed in brain, spinal cord, lymphoid tissues, nerves and ganglia of the peripheral nervous system, and in endocrine tissues in certain cases [19–21]. Accumulation, especially in retropharyngeal lymph nodes and tonsils, can also occur in the absence of spongiform lesions or clinical signs [22, 23]. Experimental oral infection of deer showed that the general progression of PrP^{Sc} accumulation is characterized by rapid and widespread involvement of lymphatic tissues followed by the tissues of the central and peripheral nervous system, the endocrine system, and the heart when animals become terminally ill [24, 25]. Early accumulation (40–45 days p.i.) of PrP^{Sc} is detected in Peyer's patches, ileocecal lymph nodes, and the retropharyngeal lymph node [22]. Approximately 90 days p.i., PrP^{Sc} deposits appear in tonsils and lymph nodes. Then PrP^{Sc} accumulation can be observed in the gut-associated lymphoid tissue (GALT) and ganglia of the enteric nervous system, as well as the dorsal motor nucleus of the vagus nerve (DMNV), the intermediolateral column of the spinal cord, and the vagus nerve. PrP^{Sc} in brain is detected first in the DMNV. From DMNV, PrP^{Sc} spreads to the rest of the brain initially affecting nuclei in the medulla, thalamus, hypothalamus, midbrain, and olfactory cortex [26]. In the late stages of infection, PrP^{Sc} deposition in endocrine tissues usually occurs [24]. Detection of PrP^{Sc} in peripheral tissues has been a valuable tool for diagnosis of CWD in cervid populations.

Deposition of PrP^{Sc} in skeletal muscle has only recently been shown [25, 27]. Accumulation of prions in skeletal muscle is of great concern to humans due to risk associated with consumption of venison from prion-infected animals. PrP^{Sc} is also found in blood, saliva, urine, feces, and antler velvet of affected deer [28–31].

This provides a plausible explanation for both lateral transmission of CWD among deer/elk and implicates contaminated environment as a likely source for oral infection.

4 Prions in the Environment

Prevalence of CWD in captive and wild herds is remarkably high and efficient horizontal transmission is driving it to epidemic proportions.

PrP^{Sc} has been shown to accumulate in a wide range of tissues in CWD affected cervids in skeletal muscle [27] and blood [28]. CWD prions have been detected in saliva, urine [32], and feces, even of asymptomatic deer [30], and cause disease as seen in transgenic cervidized mice. It has been estimated that the amount of prions excreted in feces through the course of disease is as much as is accumulated in the brain of a terminally ill deer [30].

Decomposing carcasses, feces, urine, and saliva from infected animals might be the source of contaminants in the environment and must play a role in the transmission of disease in CWD-endemic regions. There is substantial evidence that prions can exist in the environment, indicating potential reservoirs of CWD transmission [33–36]. It is well known that prions in general are not destroyed by conventional methods such as heat, chemical disinfectants, proteases, etc. To persist in soil environments, this marked stability is required to withstand extreme conditions including UV irradiation, freeze thaw cycles, extracellular enzymes from fungi and bacteria, digestion by soil inhabitants, and abiotic transformation through mineral phases [37]. Prions have been shown to adsorb onto metal and mineral deposits and are also found in aquatic environments. PrP^{Sc} shed from infected animals can cause efficient transmission when uninfected deer forage or share the same water source. Among captive cervids, prions have been detected in bedding, water, and food [38].

Environmental prions are found at relatively low levels and have to be detected by very sensitive methods. The most sensitive method available today for detection of PrP^{Sc} is the serial Protein Misfolding Cyclic Amplification (sPMCA) developed by Soto and his colleagues [39]. Another method used to detect whether body fluids are capable of transmitting disease is the inoculation of the suspected contaminant either orally or by injecting it into the body of transgenic animal models bearing cervid PrP gene [40], or inoculating or feeding PrP^{Sc} infected tissues to deer fawns [28].

In addition to body fluids and excretions, there might be other forms of discharging infectious prions [31]. Though in lower doses, the volume could be high enough to make an impact on horizontal transmission. Antler velvet of elk provides a feasible possibility for transmission. Antlers of young male cervids are covered with a highly vascularized and highly innervated apical skin layer. This velvet is shed annually due to an increase in the testosterone levels and ossification. According to this study, PrP^{Sc} was found in antler velvet, as detected by sPMCA, but in low concentrations. Though the antler velvet failed to produce disease in transgenic mice, it now represents another source of infectious prion in the environment, enabling repeated exposure to prions, though in low doses [31].

It has been demonstrated that soil may serve as an environment reservoir for prions since hamster-derived PrP^{Sc} can adhere to soil minerals [34]. Therefore, it is reasonable to argue that soil may be a source of CWD infection. Notably, oral transmissibity of infectious hamster-adapted mink prions (strains hyper and drowsy) is enhanced when bound to soil particles [41]. Though cervids do not deliberately ingest soil, they do supplement their mineral intake by licking in regions of high clay content.

There are many ways in which CWD prions could reach the soil and remain there. One possible route of soil contamination could be from decomposition of cervids infected with CWD. Though deer do not consume carcasses, they do forage on foliage that grows in abundance around the decomposition site [33]. Prolonged exposure to fecal matter could also indicate efficient horizontal transmission.

The above-mentioned routes of CWD prion contamination in soil could also lead to contamination of water due to rainfall and snow-melt run off into water sources. Due to the large number of cervids infected with CWD in the Northern Colorado area, sPMCA revealed the presence of protease resistant prion protein in the water, albeit in very low doses which could not cause infection. The authors suggest that prions could accumulate in environmental sources and could potentially promote CWD transmission [42]. Overall, it appears reasonable that environmental CWD prions may significantly contribute to horizontal transmission.

5 PrP^{c} Structure and Cervid *prnp* Gene

Amino acid sequences of *PRNP*/*prnp* genes are conserved among many mammalian species [43] as is the global three-dimensional structure of PrP^{c} (Fig. 3a, b). Therefore, this section first describes the highly conserved common structures of the cellular isoforms of PrP. The full-length PrP peptide can be divided into five regions, i.e., the endoplasmic reticulum translocation signal (ER-signal) peptide region at the N-terminus, the glycine-rich "tail" region extending over next ~80 residues, the short central domain of ~30 residues, the compactly folded globular domain, and finally the glycosylphosphatidyl–inositol (GPI) anchor attachment signal at the C-terminal extremity. As the N-terminal ER-signal peptide and the GPI-anchor signal are cleaved off in the processes of co-translational modifications, mature PrP lacks these sequences. The N-terminal tail is further divided into three regions: polybasic residues at the N-terminal end, a pre-repeat region, and five- to six-time repeats of octapeptide motif widely referred to as "octapeptide repeat region". The central domain consists of a hydrophilic so-called "charge cluster" and a highly hydrophobic region, often referred to as "amyloidogenic region" [44]. The C-terminal globular domains from different species invariably includes two short anti-parallel beta strands (β1 and β2) and three alpha helices (α1, α2, and α3) (Fig. 3b) [45]. The strikingly conserved structures of PrP^{c} imply involvement of this protein in some important physiological activities but the exact function of PrP^{c} has currently not been identified [44, 46]. After translation, PrP undergoes co- and post-translational

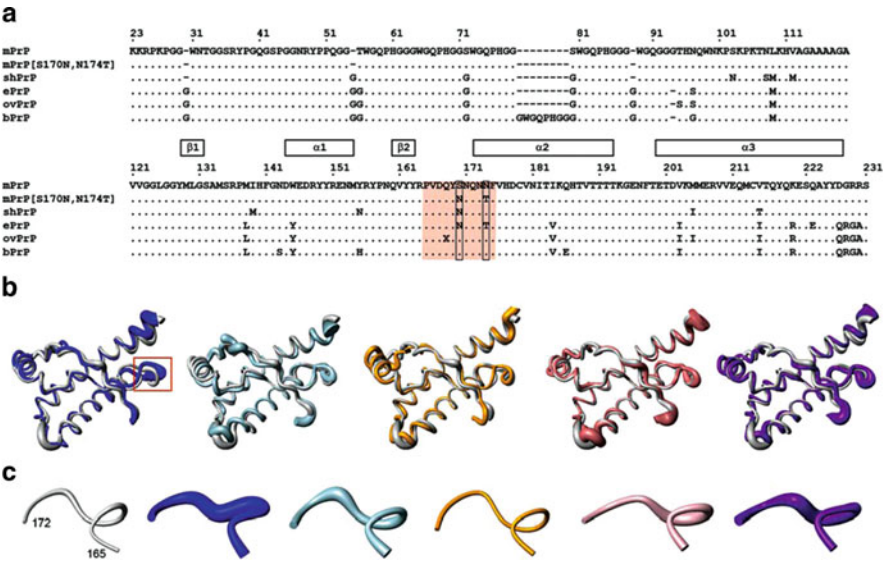

Fig. 3 Comparisons of PrP from various species, including rigid-loop mouse PrP (RL-mPrP). (**a**) PrP amino acid sequence of PrP from mouse (mPrP; *Mus musculus*) and RL-mPrP [mPrP(S170N, N174T)] and Syrian hamster (shPrP; *Mesocricetus auratus*), elk (ePrP; *Cervus elaphus nelsoni*), sheep (ovPrP; *Ovis aries*), and cattle PrP (bPrP; *Bos taurus*). Only mPrP shows all the amino acids of the residue 23–231. For the rest of the PrP, only residues different from that of mPrP are shown, while identical residues and deletions are exhibited as dots and dashes, respectively. *Boxed β1* and *β2* and *boxed α1*, *α2*, and *α3* indicate extent of β strands and α helices, respectively. The *shaded area* indicates the β2–α2 loop. "X" in ovPrP is polymorphic (Q/R). (**b**) Backbone superpositions of residues 125–228 of RL-mPrPC (*gray*) with mPrP (*dark blue*), shPrP (*light blue*), ePrP (*yellow*), ovPrP (*pink*), and vPrP (*purple*) (adapted from [4–8]). (**c**) Magnified backbone structures of residues 165–172 (indicated by the *red box* in B) in RL-mPrP, mPrP, shPrP, ePrP, ovPrP, and vPrP. The radius of the cylindrical rods is proportional to the mean global backbone displacement for residues from 20 conformers obtained by NMR analysis. The same color code is used as in (**b**). By courtesy of Dr. Adriano Aguzzi, the University Hospital of Zurich [45]

modifications, namely the removal of the N-terminal and C-terminal signal sequences, attachment of GPI-anchor along with cleavage of the C-terminal signal peptide, and N-glycosylation at two sites in the globular domain. Cervid PrP also has the highly conserved primary structure and the global three-dimensional structure, as verified by nuclear magnetic resonance (NMR) analysis [47], and undergoes the same post-translational modifications as PrP^c from other species. However, cervid PrP^c has a unique structural feature that distinguishes it from PrPs of other mammalian species. Below, the unique feature of cervid PrP is explained along with interesting phenomena derived from these investigations.

Analysis by NMR of recombinant elk PrP revealed that the loop region connecting between the second beta-strand (β2) and the second alpha helix (α2), β2-α2 loop, encompassing residues from 165 to 175 (all the codon numberings of PrP below conform numbering of corresponding residues in human PrP), was outstandingly

defined at 20 °C, whereas this region of recombinant PrP from other species, including humans, bovine, sheep, mouse, and hamsters, is usually disordered under the same conditions (Fig. 3c) [47–51]. Similar levels of well-defined loop regions were observed only in wallaby [52], bank vole [53], and horse [54]. Syrian hamster PrP showed a relatively defined loop compared with mouse PrP [51, 54]. The mechanisms that render the rigidity to β2–α2 loop seem to be different between species. In elk and bank vole, the defined loop structure was attributed to a single residue in the loop, asparagine at codon 170 [47, 53], while that of wallaby PrP was due to a long-range interaction between residue 166 in the β2–α2 loop and residue 225 in the α3 helix [52]. In the case of horse PrP, the well-defined loop is dependent on serine at codon 167 [54]. Interestingly, introduction of those residues in mouse PrP rendered rigidity even to the originally rather flexible β2–α2 loop of recombinant mouse PrP. For example, introduction of the elk residue asparagine at codon 170 (170N) and threonine at codon 174 (174T) to mouse PrP made "rigid-loop" mouse PrP (RL-mPrP) [47]. Those observations unequivocally proved that the loop rigidity is determined by those species-specific residues.

The significance of the rigid β2–α2 loop of PrP is not only of structural–biological interest. The rigid β2–α2 loop may also affect properties of PrP, especially in terms of PrP^{c}–PrP^{Sc} conversion and pathophysiology of prion infection processes. In an interspecies transmission experiment of transgenic mice overexpressing either the aforementioned RL-mPrP, which has 170N and 174T substitutions, or wild-type mouse PrP (WT-mPrP), susceptibility profiles of RL-mPrP mice to prions from other species were rather different from that of WT-mPrP overexpressing mice [45]. After inoculation of a mouse-adapted prion strain, RML, the RL-mPrP-expressing mice developed disease with an average incubation period of 323 days, whereas the wild-type mPrP expressing mice developed disease around 74 days, suggesting existence of transmission barrier in RL-mPrP mice against mouse-adapted prion. The transmission barrier was confirmed by shortened incubation periods in the secondary passage of the diseased RL-mPrP brain to other healthy individuals of RL-mPrP mice. On the other hand, RL-mPrP mice exhibited higher susceptibility to interspecies transmission from deer and hamster than WT-mPrP over-expressing mice with shorter incubation times or higher infection ratio, in spite of the expression level of the WT-mPrP being about twofold higher than that of the RL-mPrP. Interestingly, recombinant PrPs of both the species showed well-defined loop structure on NMR analysis like RL-mPrP (Fig. 3c). In contrast, no RL-mPrP mice developed disease in interspecies transmission from cattle and sheep [48, 49], whose PrP had less defined β2–α2 loop structures, whereas all WT-mPrP mice succumbed. Based on the observations and review of past literature on cross-species transmission, Sigurdson et al. postulated a hypothesis that similarity in the local structure of β2–α2 loop might be an important determinant of interspecies transmission efficiency, along with the possibility that homology in codon 170 might be especially important [45].

Even more interesting, transgenic mice expressing RL-mPrP spontaneously developed a full prion disease which was transmissible to transgenic mice overexpressing wild-type mouse PrP [55]. As deer do not spontaneously develop CWD

frequently, the pathogenicity of RL-mPrP would not be attributable solely to the rigid β2–α2 loop, and some other yet-to-be-identified genetic or environmental factors may be necessary for development of spontaneous disease. Although RL-mPrP is an artificial molecule, investigation of mechanisms by which the structural alterations influence the susceptibility profiles to various prion strains and the trigger of spontaneous conversions might shed light on PrP^{c}–PrP^{Sc} conversion mechanisms.

While the rigid β2–α2 loop is a unique structural feature, and introduction of this structure to mouse PrP induced interesting phenomena, cervid PrP itself might have a unique property. Surprisingly, transgenic mice expressing cervid PrP exhibited lateral transmission of CWD from inoculated individuals to uninoculated cage-mates, which is a most distinctive phenomenon of CWD among all prion diseases [56]. Although horizontal transmission is a characteristic of CWD and scrapie, this modality of transmission had not been observed in rodent models of experimental prion diseases. There had been a possibility that lateral transmission might be attributable to host-side factors in deer or sheep, but the observation of lateral transmission among the transgenic mice expressing cervid PrP strongly suggests that the phenomenon might be inherent in cervid PrP itself. Since such lateral transmission has not been reported for bank voles, whose PrP has as rigid a β2–α2 loop as cervid PrP, and in addition the loop of sheep PrP is not as rigid, contributions from other factors than the rigid loop might be greater for occurrence of lateral transmission of CWD. Whether horizontal transmission occurs among the transgenic mice expressing cervid PrP, even when they are infected with other prion strains, would be an intriguing question.

Thus, cervid PrP and its derivative might provide very valuable investigation materials for elucidation of PrP^{c}–PrP^{Sc} conversion and transmission barriers. Reports of further investigations are awaited.

6 Genetic Susceptibility of Cervids to CWD

In other acquired prion diseases, notably scrapie of sheep and variant Creutzfeldt–Jakob disease (vCJD) in humans, amino acids encoded at certain key positions in the endogenous host prion protein are strongly associated with susceptibility to prion infection [57–59]. Studies of CWD in elk, mule deer, white-tailed deer, and moose have found that similar correlations between PrP amino acid sequence and likelihood of CWD infection could exist in these species as well.

A study of CWD in Rocky Mountain elk [60], which have a recurrent substitution polymorphism at codon 132 encoding either methionine or leucine [61, 62], suggested that the PrP sequence at position 132 influences likelihood of infection in that species. They found that CWD-infected elk in a group of wild, free-ranging animals, and in a group of farm-raised elk from South Dakota, had a higher percentage of animals bearing the 132MM genotype than would be expected statistically, based on the frequency of methionine and leucine alleles

in comparable uninfected control populations. An over-representation of MM132 genotypes among infected elk was also reported in a captive elk population in Saskatchewan, Canada. Such findings have generated a hypothesis that the MM132 genotype predisposes exposed elk to CWD or, conversely, that the ML132 or LL132 genotype might protect them from prion disease. The idea of such a genetically created protective effect is given added credence by a similar methionine-to-valine polymorphism at the equivalent codon 129 in human PrP which has been found to be an important factor in human prion diseases [63–65]. However, since captive elk of both ML132 and LL132 PrP genotypes were found to be positive for CWD [60, 66], the ultimate susceptibility to CWD of all three genotypes has been established. Furthermore, a recent study of the PrP genotypes of 171 free-ranging elk in Colorado demonstrated the same genotype frequencies among 47 CWD infected animals as among 124 uninfected samples [67] and included one CWD infected LL132 animal. In this study, each of the three genotypes was equally susceptible to infection, a contradiction of the earlier studies that, as the authors point out, could be resolved by various differences in the sample sets of the studies, and by the long incubation time associated with the LL132 genotype as described below.

While PrP genotypes in elk thus do not confer actual resistance to CWD, lengthened incubation periods for ML132 heterozygotes and LL132 homozygous animals have been experimentally demonstrated in studies of inoculated captive elk [68, 69]. Following oral inoculation with CWD of elk of all three genotypes (2 MM132, 2 ML132, and 4 LL132 calves), clinical signs developed in both of the MM132 animals at 23 months p.i., followed by both ML132 calves at 40 months p.i. At 4 years p.i. all four of the LL132 animals showed no clinical signs, but finally succumbed at about 60 months. Whether the polymorphic PrP protein itself is directly involved in delaying the development of the disease, or whether the L132 allele segregates with other genetic factors that are closely linked to PrP propagation and accumulation, such as physiological effects or even unknown molecular and cellular pathways, has yet to be determined, but it seems clear that some protective or inhibitory effect on CWD infection of elk is created when the LL132 PrP genotype is expressed.

So far we have considered only susceptibility in natural hosts, although many studies in transgenic mice and cell free systems such as PMCA and non-natural host species such as bank voles [70] have added considerable data to the continuing effort to define clearly the rules governing control by PrP sequence of prion transmission and propagation and to reveal the molecular mechanisms at work. Most notably for CWD, by use of transgenic mice expressing either an M132 or an L132 version of the deer PrP protein, Telling's group demonstrated an inhibitory effect of the cervid PrP L132 allele on infection [71]. Transgenic mice expressing cervid PrP L132 failed to develop disease following challenge with CWD prions from any of ten different inocula from deer or elk, including an LL132 elk, although all Tg(CerPrP-M132) mice were susceptible. The Tg(CerPrP-L132) mice were infected, however, by the SSBP/1 sheep scrapie isolate. Measuring various prion strain-related characteristics, the authors determined this differential susceptibility

of M132 and L132 tg (transgenic) mice to CWD and SSBP/1 scrapie prions is a strain-dependent phenomenon. They suggest that, whatever prion strain or strains constitute CWD infectivity, the elk 132 polymorphism can restrict their propagation in tg mice, but does not inhibit propagation of the scrapie SSBP/1 strain. The implication is that the lengthened incubation times for CWD observed in LL132 and ML132 elk by Hamir et al. may result from a similar strain-dependent protective effect in the natural host, although details remain to be explored.

Telling's group have also reported an additional instance in which PrP amino acid sequence differences affect CWD transmission, using tg mice that express either deer PrP, which carries glutamine at position 226, or elk PrP, known to carry glutamate at 226 [31]. Incubation times were shorter in E226 PrP (elk) mice than in Q226 PrP (deer) mice after infection with either deer or elk sources, and histoblots showed a different pattern of prion expression in brains of the two lines of infected mice as well. Similar to their results with L132, this appears to be related to a differential ability of PrP polymorphic proteins to replicate some prion strains. The previous study was carried out with L132 tg mice that expressed Q226 rather than E226 so it is not clear what would be observed using Tg(CerPrP – L132-E226) mice in transmission studies.

Recently these researchers have identified two prevalent CWD strains (CWD1 and CWD2) which exhibit different biological characteristics, producing distinct neuropathological properties and either short or long incubation times when transmitted to tg mice that express deer PrP, but having similar conformations which are not distinguishable biochemically [72]. Interestingly, elk isolates inoculated into CerPrP tg mice produced one of two strain patterns, while transmission of deer isolates tended to produce mixtures of the two strain patterns. The authors posit that propagation of either strain by Q226 PrP-bearing deer is unstable and produces strain mixtures, while conversion of E226 PrP expressed by elk reflects relatively stable strain propagation and produces only one strain per animal which is stable upon primary passage to mice but produces mixtures after serial passage in the Q226-expressing mouse line. Based on this additional evidence of the role played by residue 226 in CWD prion strains, it will be of interest to compare strain stability of isolates from CWD infected red deer (*C. elaphus elaphus*) which are polymorphic at residue 226 and in which QQ226, QE226, and EE226 genotypes have been reported [73].

Mule deer carry a unique substitution polymorphism at codon 225 of the *prnp* gene [74, 75], which has been reported to be strongly associated with CWD disease status [76, 77]. The allele of highest frequency encodes serine, in common with most other ruminants whose gene has been sequenced. Mule deer also have an allele that encodes phenylalanine at position 225, present at widely different frequencies in free-ranging populations examined in Wyoming, Colorado, and Western Canada [76, 78]. The 225F allele was not observed in 246 samples examined from the Alberta–Saskatchewan border, while the percentage of mule deer with the 225F allele ranged from 0% to 18% in 14 herd units in Wyoming and Colorado from which samples were characterized, and as high as 22% in a different unit studied subsequently. In the Wyoming/Colorado study, *prnp* genes from 1482 free-ranging mule deer were characterized; in the total sample of 290 CWD-positive deer, only a

single 225SF individual was observed (0.3%); 22 were expected based on the overall genotype frequency (7.6%) in the sample. Despite a strong association between the 225SF genotype and low prevalence of CWD, it clearly does not confer complete resistance, as CWD-positive 225SF and 225FF deer have been detected. There is some evidence that, as in elk with a 132LL genotype, CWD incubation time in 225SF mule deer that do become infected is prolonged compared to infected 225SS deer [76]. In addition, an extensive study of the progression of PrP^{Sc} accumulation and tissue distribution in mule deer during the course of experimental CWD infection [77] produced the incidental observation that deer retroactively identified as having a 225SF genotype had displayed delayed development of disease-associated prion protein in lymphatic tissues and in the time until neuroinvasion. Studies to assess possible differences in CWD neuropathology between the 225SS and 225SF or 225FF genotypes have not been reported. Studies of susceptibility to CWD of transgenic mice expressing the mule deer PrP alleles have also not yet been reported, but it seems very possible that results similar to those obtained with the elk 132L PrP and with white-tailed deer 96S PrP (see the next section) will be observed.

As challenging as it may be to determine the mechanism by which lowered susceptibility to CWD is provided by, or is in association with, these PrP protein changes, it may be even more so to elucidate the means of action of differences in residue 20 in mule deer PrP, as reported by Wilson et al. [78]. Residue 20 falls within the signal sequence that is cleaved from the nascent PrP protein during its export from the cell interior to the outer surface of the cell membrane. Thus it is not present in the mature cellular prion protein that is affected by the disease-associated PrP during infection. These finding raise the issue of "silent" mutations also having an effect on protein tertiary structure/conformation. Slight but significant differences in PrP^{c} structure might be the effect of slower translation that resulted from codon usage differences, for instance, and could then produce strains that converted more slowly or more rapidly. Likewise, it is not totally unbelievable that the original structure of PrP^{c} could be affected by an amino acid that was later removed.

Studies of white-tailed deer *prnp* sequences and CWD disease status have been reported in Illinois [79], western Nebraska [80], Wisconsin [81, 82], and western Canada [78]. Two coding polymorphisms, Q95H and G96S, have been described which occur at lower than expected frequencies in CWD-infected animals. The 95H allele is recurrent in more than one population but rare. The serine substitution for glycine at residue 96 (G96S), although the minor allele at this position, is often observed and has been suggested to be linked to a reduced susceptibility to CWD, and slower progression of disease [82, 83]. Transgenic mice expressing cervid PrP with S96 were not susceptible to disease when inoculated with CWD pools derived from infected white-tailed deer, mule deer, or elk, while G96 transgenic mice developed disease [84]. Kelly et al. [79] also observed higher than expected frequencies of two silent polymorphisms in uninfected deer. Similar to *prnp* genetics in elk and mule deer, however, despite differences in genotypic frequencies between positive and negative animals, CWD has ultimately been identified in white-tailed deer of all genotypes. Consequently it is clear that, to explain the observations in elk and deer

reviewed here, we need to move beyond a tally of polymorphisms of the prion protein gene and examine other loci in conjunction with *prnp* allelic variations. Recently, Blanchong et al. [85] have investigated polymorphisms in complement component C1q for association with CWD susceptibility and progression, and Ernest et al. [86] have developed kinship analysis tools using microsatellite data and mitochondrial DNA sequence haplotypes that will allow finer analysis of factors influencing genetically linked transmission of CWD among white-tailed deer.

7 Natural and Experimental Transmission and Strains

CWD prions are highly contagious, at least for transmission within and between deer and elk populations. However, it is also of great interest to analyze the interspecies transmissibility or species barriers of CWD for several reasons. Such studies may enable determination of (1) the source of the CWD agent, (2) the possible distribution to carnivores feeding on infected carcasses and rodents as reservoir hosts, and (3) finally, the most important reason for such studies is to predict whether CWD might be transmissible to humans.

7.1 Natural Transmission Within Cervids

In contrast to most other prion diseases, in CWD infectivity is found in secreted and excreted products of cervids like saliva, urine, and feces [28, 30, 32]. This, and the stability of CWD prions in the environment, is most likely the reason for the remarkable horizontal transmission of CWD [38]. There is no species barrier for infections between mule deer, white-tailed deer, and elk, the species which were thought to be the only ones affected by CWD [2, 3, 14]. However, this assumption was challenged by the successful initially experimental oral transmission of CWD to moose (*A. alces shirasi*) [87] and the identification of a CWD infected free-ranging moose [1]. In order to study further distribution of CWD prions, it is important to be aware of a possible natural reservoir host, which can be rodents that cohabit with cervids in CWD endemic areas. Transmission of CWD to non-transgenic laboratory mice (*Mus musculus*) is inefficient [40], and this is also true for various hamster species [88]. However, in a recent study [89], susceptibility of four North American rodent species, including meadow voles (*Microtus pennsylvanicus*), red-backed voles (*Myodes gapperi*), white-footed mice (*Peromyscus leucopus*), and deer mice (*Peromyscus maniculatus*), to CWD upon i.c. (intracerebral) challenge was tested. All inoculated meadow voles and red backed voles, 13 of 17 white-footed mice, and 19 of 20 deer mice developed prion disease, and PrP^{Sc} was detected in their brains. These data demonstrate that natural infection of such scavenging rodents can be possible, and that these species may serve as reservoir hosts for CWD prions.

7.2 Transmission to Ruminants

The natural source of CWD has not yet been identified. One possibility is that CWD is a spontaneous disease of cervids that spreads by intraspecies transmission. Another possibility is that it arose by transmission of a prion disease from another species. Sheep scrapie was thought to be a possible source of BSE [90], and since cases of scrapie were also reported in the USA and sheep and cervids frequently share pasture, this might have led to horizontal transmission of the disease to deer and/or elk. Therefore, studies analyzing transmission of disease between sheep, goat, and cervids [91–93] or cervidized transgenic mice [71, 94] were performed. Sheep scrapie prions transmitted disease to elk upon intracerebral (i.c.) inoculation, and the induced spongiform encephalopathy was indistinguishable from CWD [92]. Diseased elk were either homozygous (MM) or heterozygous (ML) at the polymorphic codon 132 [61], indicating that the *prnp* genotype influences susceptibility of elk to scrapie prions. To analyze the influence of the polymorphism at codon 132, transgenic mice homozygous for MM or LL or heterozygous for ML were engineered and i.c. infected with CWD or scrapie. LL mice did not develop clinical disease upon i.c. inoculation with elk CWD prions similar to elk (LL) challenged with CWD prions in an oral transmission study [68]. However, LL homozygous elk also succumb to disease, albeit with a significantly prolonged incubation time compared to elk harboring at least one M allele [69]. Similarly, in aged LL tg mice subclinical levels of prions were detected [71]. However, these mice were susceptible to inoculation with SSBP/1 scrapie prions, indicating that the partially protective role of the polymorphism at codon 132 is dependent on the prion strain [71]. Transmission of CWD prions can be performed via i.c. inoculation to both goat [91] and sheep, although in case of sheep with only low efficiency [93]. In the latter study, the influence of the ovine *prnp* genotype, which dictates development of scrapie in sheep, on susceptibility to CWD was investigated. Interestingly, susceptibility to infection and clinical scrapie was influenced by the *prnp* genotype, similar to what can be observed for scrapie in sheep [93]. In both sheep and goat, spongiform encephalopathy and disease upon CWD infection resembled sheep scrapie [91, 93]. Also comparison of glycoform patterns of PrP^{Sc} derived from CWD infected deer and elk or scrapie infected sheep revealed a high degree of similarity between the agents [95].

For transmission studies and analysis of species barrier, transgenic mouse models have been proven to be valuable tools [96, 97]. Since mice are not susceptible to infection with CWD, tg mouse models overexpressing cervid PrP have been engineered by several groups [40, 71, 98, 99]. Tamgüney et al. [94] employed tg mice overexpressing elk PrP to study susceptibility to scrapie and BSE prions. One isolate (SSBP/1) of scrapie induced disease in those mice whereas another isolate (027) derived from a North American case of scrapie did not. The tg mice were not susceptible to infection with cattle-derived BSE prions; however, upon passage of BSE through sheep, the disease could be transmitted, although with a slightly longer incubation time compared to the sheep scrapie isolate.

The inability to transmit BSE prions contrasts with a study demonstrating successful inoculation of European red deer (*C. elaphus elaphus*) with BSE. This species is closely related to elk and is also highly susceptible to CWD [10].

In transmission studies to ruminants, a special focus has been placed on inoculation of cattle. Since BSE crossed the species barrier to humans there is concern that CWD might become more infectious to humans upon passage through cattle. In an initial study CWD derived from mule deer was used for i.c. inoculation of cattle; however, only a low number of the inoculated animals (38%) tested positive for PrP^{Sc}, with only very mild clinical symptoms, a long incubation period, and absence of lesions typical for spongiform encephalopathy [100]. Brain homogenates of one of the PrP^{Sc}-positive cattle were used for second passage in cattle. In second passage cattle, incubation time was shortened and clinical signs were observed in five out of six animals. Lesions associated with prion disease were absent, although PrP^{Sc} was detected by IHC and immunoblot [101]. Using white-tailed deer-derived CWD as an inoculum, the frequency of transmission was much higher with 92% of the inoculated animals demonstrating clinical signs. As with mule deer CWD, no spongiform lesions were present in the CNS despite detectable PrP^{Sc} [102]. The authors suggest that absence of spongiform lesions might offer a possibility to distinguish between CWD and BSE in cattle. Altogether, these studies demonstrate that CWD prions can be transmitted to cattle at least upon i.c. inoculation, although efficiency appears to be dependent on the species the prions are derived of.

7.3 Transmission to Carnivores

CWD is mainly a disease of free ranging animals, raising concern for transmission to predators that feed on carcasses of infected cervids harboring infectious prions. For instance, observations of the feeding behavior of mountain lions, which can be affected by BSE-derived prion disease [103], revealed that they prey preferably on CWD infected deer [104]. Unfortunately, published experimental data on oral transmission data to mountain lion and other predators of cervids such as gray wolves, coyotes, or bears are still lacking, but studies on other carnivores indicate a strong species barrier. This is supported by a recent surveillance of scavengers (mink, raccoons, coyotes) feeding on white-tailed deer carcasses in a CWD endemic area in Wisconsin [105]. Tissues of those carnivores were collected and analyzed for presence of PrP^{Sc} but none of the samples tested positive. To study experimentally further species barrier properties, ferrets were infected both i.c. and orally with mule deer derived CWD prions, and oral transmission was not successful whereas i.c. inoculation led to disease, although with a long incubation time [106]. Similar results were obtained when minks were employed in oral and i.c. transmission studies with elk-derived CWD, which again were only susceptible upon i.c. inoculation with a long incubation time [107]. Racoons were resistant even to i.c. inoculation [108].

7.4 CWD Strains

The occurrence of different CWD strains, which are characterized by different incubation periods, diverse brain regions affected by spongiform lesions, or the glycoform pattern of PrP^{Sc}, was unclear for many years. Transgenic mice expressing cervid PrP were used for inoculation with elk or mule deer derived CWD prions, and in this model no evidence for CWD strains was revealed [40]. In contrast, when elk PrP expressing tg mice were inoculated with mule deer or elk CWD, differences in incubation time, clinical disease, and histopathology indicated the existence of different CWD strains in primary infected mice and upon passage [99]. In a recent study, the existence of at least two distinct CWD strains (CWD1 and CWD2) has been confirmed [72]. Interestingly, deer appear to harbor a mixture of CWD1 and CWD2, whereas elk are affected by either CWD1 or CWD2, which may be dictated by differences in the primary structures of deer and elk (Q226 vs E226; [72]). However, it is not clear by what means prion propagation in elk is restricted to either one strain or the other if infected with a mixture of CWD1 and CWD2, such as would be present in an inoculum from mule deer. Whether this reflects a stochastic choice of dominant path early in amplification of a self-propagating strain, or an incompatibility between the two strains that does not allow their coexistence in elk, are among issues remaining to be investigated. In light of these findings, the different stabilities of those strains, and the influence of residue 226, it will be important to re-assess tissue distribution and zoonotic potential of these isolates.

8 Zoonotic Potential

Deer hunting is a popular sport in the USA; venison is usually consumed by hunters and their families, and this raises reasonable concerns about the transmissibility of CWD to humans, as exemplified by the zoonotic transmission of BSE. Epidemiological studies have not revealed an increased incidence of CJD in CWD endemic areas. Suspicious case reports about persons having consumed venison and succumbed to neurological disorders turned out to be classical or familial CJD and a causal link to the consumption of contaminated meat could not be proven [109]. In vitro conversion assays were performed to assess the convertibility of human PrP^{c} into PK resistant PrP using CWD derived from elk, mule deer, and white-tailed deer as a template. Only a very low conversion rate could be demonstrated, indicative of a considerable species barrier [110]. In a very recent study employing protein misfolding cyclic amplification (PMCA), a highly sensitive method for in vitro amplification conversion of human PrP^{c} was successful, albeit with the restriction that CWD prions had to be adapted by PMCA or in vivo passage through cervidized tg mice. Passaged CWD template gave rise to a new form of human PrP^{Sc}, and its infectivity is currently under investigation. Of note, when field isolates of CWD

brains were used as a template, no conversion of human PrP^c was achieved, which again questions the relevance of the in vitro data [111]. Studies using humanized tg mouse inoculated with CWD furthermore argue against transmissibility to humans since mice did not develop prion disease [112–114]. The best possible animal models to study transmission of animal prion diseases to humans probably are non-human primates. For instance, inoculation of BSE prions into *Cynomolgus* macaques provided the first hints that BSE might be transmissible to humans [115]. However, oral or i.c. challenge with CWD of macaques did not cause disease [116]. On the other hand, squirrel monkeys (*Saimiri sciureus*) were susceptible to i.c. inoculation with CWD prions [116, 117], and after oral exposure PrP^{Sc} was detectable in brain, spleen, and lymph nodes of 2/15 squirrel monkeys [116]. This shows that CWD can be transmitted to certain non-human primates, although results in *Cynomolgus* macaques might be of higher relevance since they are evolutionary closer relatives of humans than squirrel monkeys. Overall, there is no compelling evidence that CWD can be transmitted to humans with high efficiency.

9 CWD Diagnostics

Diagnosis based on clinical symptoms is not reliable, as clinical signs are unspecific and mild at the beginning of disease. Later, the leading symptom is extensive and progressive weight loss (wasting), often accompanied by hypersalivation, polydipsia, ataxia, and behavioral signs like isolation from the herd. Post-mortem surveillance is based on testing of brain material (i.e., obex region) for PrP^{Sc} by immunohistochemistry (IHC) and commercial ELISA or immunoblot tests, in analogy to BSE testing. Although regionally varying, the two main strategies are targeted surveillance and hunter harvest-based surveillance. The former actively targets animals with symptoms consistent with clinical CWD for harvest and subsequent CWD testing. The latter represents the systematic sampling and testing of hunter-killed cervids, with the caveat that testing is voluntary. Hunters whose donated heads were tested positive are informed, but turnaround time can be as long as 12 weeks. As many weeks may elapse between harvest and final test result, this strategy does not guarantee that human consumption of CWD prion containing materials is prevented, and many hunters may choose not to participate. Passive collection and testing of natural mortalities and roadkills is usually done in parallel. Overall, such strategies are very useful for acquiring epidemiological data on prevalence but do not protect the consumer a priori.

Therefore, for wildlife management and containment purposes rapid and versatile intra vitam tests are needed. Given the wide distribution of CWD prions in the animal's body, antemortem sampling and testing of accessible lymphatic tissues such as tonsils, retropharyngeal lymph nodes, and rectal biopsy materials was found to be very predictive in pre- and sub-clinical animals. PrP^{Sc} is then usually detected

by IHC and such testing reaches high sensitivity rates, although not as sensitive as using brain materials [118].

Another feature of CWD which might be useful in diagnosis is the heavy presence of CWD prions in body fluids (blood, CSF) and secretions (saliva, urine). Prion/PrP^{Sc} amplification methods based on seeding/conversion (work of Byron Caughey and colleagues) or cyclic amplification of protein misfolding (PMCA, work of Claudio Soto and colleagues) have increasingly reached extremely high sensitivity, good versatility, and decent specificity and robustness. Although the latter still need significant improvement and simplification before becoming applicable in routine diagnostics, such techniques are presently the most promising candidates for live testing for prion diseases, including CWD [32, 119–121].

Another strategy for developing live testing aims at surrogate markers, usually proteins, which are up- or down-regulated as a specific response to prion infection and/or prion-induced neurodegeneration. Although such tests are routinely used in diagnostics of human prion diseases from cerebro-spinal fluid (CSF; e.g., NSE, 14-3-3), CSF is not a viable option for animal prion infections. In case of CWD, materials like blood, urine, or even feces may be more approachable. Some groups also aimed at increasing both specificity and sensitivity of existing diagnostic test formats towards ultrasensitive immunoassays, although without great success [122]. Use of antibodies postulated to be specific for PrP^{Sc} might be another option [123]. Finally, for CWD diagnosis in particular, there is the tremendous problem of tracking wild life and having access to biological materials without using invasive methods that would involve the need of anesthesia.

10 Conclusions and Future Challenges

Control of CWD is presently impossible and its eradication in future is unlikely. This is the only prion disease which is naturally occurring in free-ranging and non-domestic animals which are shy and migrate over vast distances. Other unique features of CWD are the highly pronounced horizontal spread and the very likely involvement of environmental factors in transmission and the preservation of the infection chain. Although affected captive herds can be depopulated, culling is not a realistic option in wild-living animals. Therefore, combined efforts involving wild-life disease management, public awareness, basic research, and translational research will be needed to define strategies for containment, possibly in the form of wild-life vaccines, and to develop practical live animal tests that are affordable and provide rapid intra vitam diagnosis. Intra- and inter-species restrictions in transmissibility, the possible involvement of intermediate hosts, and environmental, genetic and epigenetic co-factors have to be understood in greater detail. In the short term, we must prevent CWD being introduced to caribou herds in the extreme Northern parts of America, which are particular vulnerable as they assemble in very large and densely packed migratory herds and serve as a major food source for Native Americans. Although presently available experimental and epidemiological data do not favor a likely zoonotic

potential of CWD, it might be prudent to focus present-day more on containing the spread and prevalence of CWD in order to minimize the potential infectious load for human consumption. There was a time BSE was considered unlikely to transmit to humans and vCJD was only recognized because its clinical presentation was so different from that of classic CJD.

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Transgenic Mouse Models and Prion Strains

Glenn C. Telling

Abstract Here we review the known strain profiles of various prion diseases of animals and humans, and how transgenic mouse models are being used to elucidate basic molecular mechanisms of prion propagation and strain variation and for assessing the zoonotic potential of various animal prion strains.

Keywords Quasi-species · Species barriers · Strains · Transgenic models

Contents

G.C. Telling (✉)
Sanders Brown Center on Aging, University of Kentucky Medical Center, Lexington, KY 40506, USA
and
Department of Microbiology, Immunology and Molecular Genetics, University of Kentucky Medical Center, Lexington, KY 40506, USA
and
Department of Neurology, University of Kentucky Medical Center, Lexington, KY 40506, USA
e-mail: gtell2@uky.edu

1 Prion Strains, Species Barriers, and Transgenic Mouse Models

A crucial aspect of prion disorders is their transmissibility. Inoculation of diseased brain material into individuals of the same species will typically reproduce the same disease, but with prolonged, clinically silent incubation periods. Understanding the mechanism by which a protein-only infectious agent encodes information required to specify distinct disease phenotypes, and how such strains mutate and acquire fitness in the absence of nucleic acids, is central to the mechanism of protein mediated replication. Because strain properties are a crucial determinant of prion species barriers, understanding these aspects is crucial for implementing control strategies, and for evaluating the zoonotic potential and the general host range of prions.

Characterization of prion strain properties relies mainly on experimental transmissions of isolates in animal models, an isolate referring to a primary source of prion infectivity from a naturally occurring disease. As such, isolates may contain one or more "strains," which generally refers to infectivity for which distinctive phenotypic properties have been experimentally characterized in animal models. Standard criteria for characterizing and differentiating strains include the distribution and severity of PrP-associated pathology, often revealed by staining brain sections with anti-PrP antibodies [1], and the time to onset of disease after inoculation, referred to as the incubation time [2, 3]. Several serial passages within a host species of invariant PrP genotype are usually required to establish the phenotypic properties of strains. Strain cloning is generally accomplished by subjecting a strain to serial passage at limiting dilution, with the aim of purification from minor, slower replicating strains.

The initial framework for understanding prion strains is derived from studying the phenotypic properties of rodent-adapted sheep scrapie isolates by intracerebral inoculation of inbred wild type mouse lines with diseased brain extracts, and determining neuropathological characteristics and the incubation period to clinical disease. Once adapted, and using consistent doses and routes of inoculation, these strains were shown to have remarkably consistent properties, reflected in incubation times with standard errors generally $<2\%$ of the mean when serially passaged in mice of defined genotype [4].

Subsequent studies revealed the gene encoding PrP, referred to as *Prnp*, as the most important host factor controlling incubation times in mice [5]. The incubation period of strains is influenced by allelic variation of the murine PrP gene. Two alleles (designated a and b) encode proteins that differ at codons 108 [leucine (L) for genotype a mice, phenylalanine (F) for genotype b mice], and 189 [threonine (T) for genotype a mice, valine (V) for genotype b mice]. Notwithstanding a general lack of inbred lines, strain typing has proven to be feasible in other species. In particular, hamsters and bank voles have proven useful for differentiating several prion strains [6, 7].

By varying transmission conditions, early strain typing studies in wild type mice identified three classes of strains with varying stabilities [2]. While the properties of class I strains are stable across genotypes, class II strains are stable when passaged in the *Prnp* genotype in which they were isolated, but gradually change during serial passage in another genotype until achieving a new set of stable properties. Class III strains are stable when passaged at high dilutions; otherwise strain properties break down, giving rise to new properties.

Despite these early advances, the use of inbred wild type mice for strain typing is associated with several drawbacks. One is the generally inefficient propagation of human and animal prion isolates in mice. Such examples of inefficient trans-species prion transmission are referred to as species barriers [8]. The transmission barrier may be absolute in which case no transmission is recorded, or partial, in which case primary transmission is either characterized by long incubation times and low attack rates followed by greatly-reduced incubation times and high attack rates on secondary passage, or by high attack rates on both primary and secondary passage with reduced incubation times on secondary passage. Second, since multiple prion strains may exist in a single isolate, and host genetic background can influence strain characteristics, when a strain moves from one species to another, strain characteristics can alter in unpredictable ways [9]. Interspecies transmission may therefore result in selection of minor strains that may not represent the dominant populations of strains in the original inoculum.

Although the elements controlling interspecies prion transmission are not completely understood, seminal studies in transgenic mice [10, 11] and in cell-free systems [12] indicated that the sequences of PrP^{Sc} in the inoculum and PrP^{C} in the host should be closely related for optimal progression of disease. The influence of intra-species PrP polymorphisms on prion disease susceptibility in mice [5], sheep [13], and humans [14–16] supported the importance of PrP primary structure. In seminal experiments, Scott and co-workers abrogated the resistance of mice to hamster prions by transgenic expression of hamster PrP^{C} in mice [17]. The lack of species barrier during homotypic transmission, i.e., when the host expresses a PrP gene identical to that of the infecting species, has led to the development of many different facile mouse models in which to study the biology of mammalian prions, including sheep, bovine, human, cervid, and mink, by transgenic expression in mice of PrP coding sequences from these various species. Generally, an inverse correlation exists between the length of prion incubation time in these transgenic mouse models and transgene expression level. Transgenes are usually expressed on a *Prnp* knockout background ($Prnp^{0/0}$) [18] in order to avoid partial or full suppression of disease caused by co-expression of wild type PrP. This inhibitory effect was first observed during the characterization of transgenic mice expressing human PrP which only became susceptible to CJD prions when endogenous wild-type mouse PrP was eliminated by crossing the transgene array to $Prnp^{0/0}$ knockout mice [19, 20]. Inhibitory interactions of wild type mouse PrP have also been shown to modulate neurodegeneration in transgenic mouse models expressing PrP with a mutation associated with the inherited human prion disease Gerstmann Straussler Scheinker (GSS) syndrome [21]. Interestingly, and in contrast to the GSS

transgenic model, neurodegeneration induced by expression of a nine-octapeptide insertion associated with familial CJD, designated Tg(PG14), is unaffected by wild-type mouse PrP expression [22].

A variety of different transgenic mice expressing chimeric versions of PrP in which specific regions of mouse PrP primary structure were replaced by the corresponding elements from human, sheep, and bovine PrP have also been created [19, 20, 23–25].

The aforementioned drawbacks of transmitting human and animal prions to wild type mice have been largely circumvented by transgenic approaches. Increasingly, such models are now used for typing strains in human and animal prion isolates, and have also provided valuable mechanistic information about prion propagation [26]. In an alternative approach, expression of foreign PrP genes in mice has been accomplished by gene replacement methods. This approach ensures that the PrP coding sequence is controlled by the same regulatory elements as wild type mouse PrP, in which case gene expression is expected to recapitulate authentic PrP^C expression. While microinjection and gene replacement models generally provide complimentary results, transgenic overexpression is desirable in most cases since it results in highly reduced incubation times to assess fully the extent of a species barrier. For example, while human PrP knock-in mice did not register disease when challenged with prions from cattle affected with bovine spongiform encephalopathy (BSE) [27], BSE infection did occur in transgenic mice expressing human PrP [28]. Strain typing methods developed with both kinds of transgenic mouse models are essentially similar to those used in wild-type mice.

2 Scrapie in Sheep and Goats

The precise number of strains existing in field isolates of scrapie affected sheep and goats remains uncertain. However, given the aforementioned influence of PrP primary structure on strain properties, the existence of at least 26 major *PRNP* polymorphisms in sheep is likely to influence assessments of sheep scrapie strain diversity. Scrapie susceptibility is strongly influenced by PrP gene (*PRNP*) polymorphisms. Three major polymorphisms in sheep PrP correlate with susceptibility to scrapie [13] V or alanine (A) at position 136, arginine (R) or histidine (H) at position 154, and glutamine (Q), R or H at position 171 [29–31]. The most susceptible genotypes are VRQ/VRQ, ARQ/VRQ, and ARQ/ARQ, whereas homozygous or heterozygous AHQ and heterozygous ARR animals are relatively resistant and ARR/ARR sheep are strongly resistant [30, 32].

As mentioned previously, assessment of scrapie strain diversity initially relied on transmission of natural isolates to inbred mice [33]. From such an approach, estimates of up to 20 strains were described [34, 35]. However, more recently, this estimate has been revised to three distinct strains, namely ME7 and 87A in short incubation time *Prnp-a* mice and 87V in long incubation time *Prnp-b* mice [4, 33, 36].

In recent years, a so-called "atypical" form of scrapie has been discovered through active surveillance of European flocks by rapid detection of PrP^{Sc} in brain tissues at the abattoir/slaughterhouse [37, 38]. The term classical scrapie is often used to distinguish previously recognized forms from atypical scrapie. Atypical scrapie was first reported in Norwegian sheep in 2003 and was referred to as Nor98 [37]. Because regulatory requirements implemented in 2002 led to intensified scrapie surveillance across the European Union, and because of improvements in PrP^{Sc} detection methods, additional European countries also identified TSE-infected sheep with characteristics similar to the so-called Nor98 scrapie isolate. Atypical scrapie became the term applied to cases producing discordant responses in the rapid tests applied to large-scale random screening of slaughtered or fallen animals. Precautionary testing methods revealed surprising numbers of atypical scrapie cases in Europe [39]. By 2005 atypical cases in Great Britain represented 37% of total scrapie cases in sheep.

Atypical and classical forms of scrapie differ in a number of important respects. Sheep with atypical scrapie are invariably older, and cases are frequently only found in single animals in an infected flock [40]. Unlike the pathology of classical scrapie, there is little or no neuronal vacuolation or immunohistochemically-detectable PrP^{Sc} in the obex region of the brain with atypical scrapie cases [37]. Abnormal PrP in atypical cases has been shown to be more susceptible to enzymatic digestion than in classical scrapie [41]. Also, characterization of protease-resistant PrP^{Sc} by Western blot shows a unique biochemical signature that is shared by all atypical scrapie cases, most prominently, a characteristic band of apparent low molecular weight 12 kDa that is not seen in analyses of classical scrapie cases [37]. Finally, atypical scrapie occurs in sheep with *PRNP* genotypes usually associated with resistance to classical scrapie, particularly with the AHQ allele. Sheep with atypical scrapie are more likely to express phenylalanine (F) than leucine (L) at codon 141 [40].

The use of transgenic mice expressing ovine PrP (OvPrP), which lack a transmission barrier to scrapie, and bank voles, which are particularly susceptible to sheep prions, is leading to a more comprehensive view of scrapie strain diversity. Transgenic mouse models also offer flexible and tractable systems in which to study scrapie strains, as well as the effects of *PRNP* polymorphisms on scrapie susceptibility. Transgenic mice expressing the OvPrP coding sequence with ARQ or VRQ at codons 136, 154, and 171 have been developed to study sheep scrapie prions [42–44]. Transmission of about 80 classical scrapie isolates from Europe has so far resulted in categorization into four phenotypically distinct classes [41, 44–46]. The behavior of sheep scrapie isolates in transgenic mice expressing the OvPrP ARQ allele [43] also suggests the existence of strains with distinct biochemical properties of PrP^{Sc} [47, 48]. Current evidence suggests that atypical scrapie may be a single, unique strain [38, 41]. While atypical scrapie cases do not transmit to wild type mice, these prions propagate without an apparent species barrier in transgenic mice overexpressing the sheep VRQ allele [41].

While clinical and pathological observations of natural goat scrapie also indicate the existence of multiple field isolates, strain characterization of prions from goats

has been relatively limited. Passage of the SSBP/1 sheep brain pool into goats resulted in two phenotypes referred to as "drowsy" and "scratching," and subpassage into mice of isolates from these two goat phenotypes resulted in different strains [35]. Notably, goats with atypical scrapie [41] and BSE [49, 50] have been identified in Europe through active surveillance programs.

3 Prion Strain Diversity in Cattle

While BSE initially appeared to be a homogeneous disease, the large-scale testing of livestock nervous tissues for the presence of PrP^{Sc} led to the recognition, in Europe, Japan, and the USA, of two additional bovine PrP^{Sc} variants termed H- and L-types [51]. The molecular signature of bovine PrP^{Sc} from animals with the bovine amyloidotic spongiform encephalopathy (BASE) variant corresponds to L-type, and appears similar to a distinct subtype of sporadic CJD (sCJD) [52]. L-type has a tendency to form amyloid plaques in cattle brain and has a distribution of brain pathology distinct from BSE [52]. These "atypical BSE" cases have been detected in aged asymptomatic cattle during systematic testing at the slaughterhouse. The etiology of these atypical forms remains unexplained but could involve either (1) a change in the biological properties of the BSE agent, (2) infection of cattle with prions from another source, such as scrapie or CWD, or (3) previously unrecognized sporadic forms of prion disease in cattle. A case of BSE in the US with an H-type PrP^{Sc} signature in an approximately 10-year old cow from Alabama was also associated with mutation of glutamate (E) to lysine (K) at codon 211, referred to as E211K [53]. Of particular significance, the identical substitution at the equivalent codon 200 in human *PRNP* is linked to the most frequent form of familial CJD.

The development of various transgenic mouse models expressing bovine PrP resulted in valuable experimental models for characterizing and titrating BSE infectivity [25, 54, 55], and provided compelling evidence for a relationship between vCJD and BSE [56]. While these transgenic mouse models were characterized by rapid incubation times and 100% attack rates, mice expressing bovine PrP generated by gene replacement of the mouse PrP coding sequence had long incubation times (>500 days) and incomplete attack rates [27]. The experimental transmission of H- and L-type cases to bovine PrP transgenic mice unambiguously demonstrated their infectious nature and revealed strain properties distinct from BSE [57–60]. Strikingly, serial passage of the L-type strain to wild type mice, and mice expressing the VRQ allele of OvPrP, induced a disease phenotype indistinguishable from that of BSE [57, 60], suggesting a possible etiological relationship between atypical and classical BSE. The relevance of these findings to studies in transgenic mice, which consistently reveal the existence of more than one molecular type of PrP^{Sc} [28, 61, 62] and suggest that more than one BSE prion strain might infect humans [28], remains to be determined.

Challenge of two lines of transgenic mice expressing human PrP with M at codon 129 with L-type isolates produced a molecular phenotype distinct from classical BSE [63, 64]. In one case, L-type transmitted with no transmission barrier [63], and in both cases the L-type PrP^{Sc} biochemical signature was conserved upon transmission. In contrast, the transmission efficiency of classical BSE and H-type isolates to transgenic mice expressing human PrP is relatively low [28, 63, 65].

4 Prion Strain Diversity in Cervids

CWD is transmissible after intracerebral inoculation of mule deer with incubation periods of up to 2 years [66]. Experimental transmission to other species has had mixed results. Wild type mice appear resistant to CWD infection [67], and there is also a species barrier to transmission of CWD to hamsters, ferrets, and mink [9, 68, 69].

The last few years have witnessed the development of several transgenic mouse lines expressing either elk or deer PrP in which the species barrier to CWD has been eliminated. Prototype transgenic mice expressing deer PrP, designated Tg(CerPrP) $1536^{+/-}$ [67], recapitulated the cardinal neuropathological, clinical, and biochemical features of CWD, an observation subsequently confirmed in comparable transgenic mouse models expressing deer or elk PrP [70–76]. The presence of CWD prions in saliva, blood, urine, and feces [77–80] is consistent with the mechanism of contagious lateral transmission. The detection of CWD prions in elk antler velvet by transgenic bioassay, and the annual shedding of this material, raises the possibility that it may also play a role in CWD transmission [70].

As demonstrated in other species in which prion diseases occur naturally, susceptibility to CWD is highly dependent on polymorphic variation in deer and elk *PRNP*. In mule deer, polymorphism at codon 225 encoding serine (S) F influences CWD susceptibility, the 225F allele being protective. The occurrence of CWD was 30-fold higher in deer homozygous for S at position 225 than in heterozygous animals; the frequency of 225 S/F and 225 F/F genotypes in CWD-negative deer was 9.3%, but only 0.3% in CWD-positive deer [81]. Polymorphisms at codons 95 (Q or H) [82], glycine (G) or S at codon 96 [82, 83], and A or G at codon 116 [84] in white-tailed deer have been reported. While all major genotypes were found in deer with CWD, the Q95, G96, A116 allele (QGA) was more frequently found in CWD-affected deer than the QSA allele [82, 85]. The elk *PRNP* coding sequence is also polymorphic at codon 132 encoding either M or leucine (L) [86, 87]. This position is equivalent to human *PRNP* codon 129, which is also polymorphic and encodes either M or V. Studies of free-ranging and captive elk with CWD [88], as well as oral transmission experiments [89, 90], indicate that the 132 L allele protects against CWD.

To address more fully the influence of the elk 132 polymorphism, transmissibility of CWD prions was assessed in transgenic mice expressing cervid PrP^{C} with L or M at residue 132 [71]. While transgenic mice expressing CerPrP-L132 afforded partial resistance to CWD, SSBP/1 sheep scrapie prions transmitted efficiently to

Tg(CerPrP-L132) mice. These findings suggested that the elk 132 polymorphism controls prion susceptibility at the level of prion strain selection. The contrasting ability of CWD and SSBP/1 prions to overcome the inhibitory effects of the CerPrPL132 allele is reminiscent of studies describing the effects of the human codon 129 methionine M/V polymorphism on vCJD/BSE prion propagation in transgenic mice, which concluded that human PrP V129 severely restricts propagation of the BSE prion strain [62]. Resistance to CWD was also reported in transgenic mice expressing serine at residue 96 [74].

While strain diversity is well documented for sheep scrapie, BSE, and human prions, until recently the prevalence of cervid prion strains had not been assessed. Although original studies in transgenic mice [67] and subsequent work [73] raised the possibility of CWD strain variation, the limited number of isolates and the lack of detailed strain analyses in those studies meant that this hypothesis remained speculative. Subsequent studies supported the feasibility of using Tg(CerPrP) $1536^{+/-}$ mice for characterizing naturally occurring CWD strains, CWD prions generated by protein misfolding cyclic amplification (PMCA), and novel cervid prions [91]. The prevalence of CWD prion strains in a large collection of captive and wild cervids from different species and geographic locations was assessed by bioassay in transgenic mice [92]. The findings provided substantial evidence for two prevalent CWD prion strains, referred to as CWD1 and CWD2, with different clinical and neuropathological properties. Remarkably, primary transmissions of CWD prions from elk produced either CWD1 or CWD2 profiles, while transmission of deer inocula favored the production of mixed intra-study incubation times and CWD1 and CWD2 neuropathologies. These findings indicate that elk may be infected with *either* CWD1 *or* CWD2, while deer brains tend to harbor CWD1/CWD2 strain mixtures.

The different primary structures of deer and elk provides a framework for understanding these strain profiles and the hypothesis that CWD strain mutation is governed by the relative stabilities of prion propagation by elk and deer PrP primary structures. Thus, propagation of either strain by $CerPrP^{C}$-Q226 in deer brain is unstable, and both strains are manifest as mixtures; the almost exclusive manifestation of CWD1 or CWD2 strains following primary transmissions of elk CWD prions reflects relatively stable strain propagation by $CerPrP^{C}$-E226 in elk. Supporting this interpretation, serial passage of prions in the brains of diseased Tg (CerPrP)$1536^{+/-}$ mice produced strain mixtures, regardless of whether those prions originated in diseased elk or deer. Mutation of CWD1 and CWD2 propagated by $CerPrP^{C}$-Q226 is reminiscent of unstable (Class III) scrapie strains such as 87A, and its more stable counterpart, ME7 [34], but with CWD1 and CWD2 representing a novel strain class, with neither being a more stable derivative of the other. Because of the role played by residue 226, the description of a lysine (K) polymorphism at this position in deer [93], and its possible role on strain stability, may also be significant.

It is unknown whether CWD1 and CWD2 interfere or act synergistically, or whether their co-existence contributes to the unparalleled efficiency of CWD transmission. Interestingly, previous CWD transmission studies in transgenic

mice suggested that cervid brain inocula might be composed of strain mixtures [75]. Additional previous studies also support the existence of multiple CWD strains. CWD has also been transmitted, albeit with varying efficiency, to transgenic mice expressing mouse PrP [75, 94]. In the former study, a single mule deer isolate produced disease in all inoculated Tga20 mice. On successive passages, incubation times dropped to ~160 days. In the second study, one elk isolate from a total of eight deer and elk CWD isolates induced disease in 75% of inoculated Tg(MoPrP)4053 mice. It is worth noting that the distribution of lesions in both studies appeared to resemble the CWD1 pattern. Low efficiency CWD prion transmission was also recorded in hamsters and transgenic mice expressing Syrian hamster PrP [95]. In that study, during serial passage of mule deer CWD, fast and slow incubation time strains with different patterns of brain pathology and PrP^{Sc} deposition were also isolated.

Whether the host range of CWD extends beyond the family Cervidae is currently unclear. However, the remarkably high rate of CWD prion transmission brings into question the risk posed to livestock from developing a novel CWD-related prion disease via shared grazing of CWD contaminated rangeland. This issue has been indirectly addressed by transmitting CWD to transgenic mice expressing ovine or bovine PrP, with negative outcomes [75]. Surveillance currently shows no evidence of CWD transmission to humans [96, 97]. While CWD prions have hitherto reassuringly failed to induce disease in transgenic mice expressing human PrP [72, 75, 98], CWD transmission to certain species of non-human primates has been reported [68, 99].

5 Transmissible Mink Encephalopathy

Transmissible mink encephalopathy (TME) is a rare disease affecting farmed mink. Since 1947, there have been 5 outbreaks on 11 ranches in the United States, 3 occurring in Wisconsin [100]. Outbreaks were also reported in East Germany, Finland, and the USSR in the mid-1960s. The etiology of TME is unclear; however, like BSE, food-borne infection is the most convincing explanation since outbreaks occurred in mink production facilities that prepared on-site feed involving the use of non-ambulatory cattle [101]. While neuropathological and clinical features of scrapie-infected mink are similar to mink with natural and experimental TME [102], transmission is inefficient and mink are not susceptible to oral infection with scrapie [103]. Mink also have a species barrier to infection with CWD [104]. However, transmission to cattle of TME from the Stetsonville, Wisconsin outbreak resulted in disease after 18.5 months, and re-transmission to mink showed that infectivity was not significantly altered by passage in cattle [101]. In other studies, bovine adapted TME and TME from mink, when passaged to cattle, produced indistinguishable incubation times and similar neuropathological profiles [105]. Collectively, these studies substantiate the hypothesis that bovine prions were the source of TME infection. There is, however, no evidence for a causative link with

classical BSE: there is no teleological overlap between BSE and TME, and the disease characteristics of BSE-infected mink are different from TME [100]. Moreover, the disease profile of cattle inoculated with TME differs from classical BSE [105, 106].

In a provocative study, the transmission profile of the bovine-passaged Stetsonville TME isolate was compared with classical BSE and H- and L-type bovine prions in transgenic mice expressing the ARQ OvPrP allele. The phenotypic properties of L-type and bovine TME prions were similar in these mice, suggesting a possible relationship between L-type bovine prions and TME. Although L-type prions have not been detected in US cattle, active surveillance measures for bovine prion disease only target 40,000 high-risk cattle per annum. One interpretation of the outbreaks of TME in the US, Eastern Europe, and the USSR, is that mink are sentinels for L-type bovine prions which occur at low levels in cattle populations, possibly sporadically. Given that L-type bovine prions transmit to transgenic mice expressing human PrP with considerable efficiency [63], these results would appear to have considerable relevance to public health.

The transmission barrier of TME prions in mice was abrogated by transgenic expression of mink PrP on a knockout $Prnp^{0/0}$ background [107].

6 Human Prion Strain Diversity

The human prion diseases uniquely manifest as sporadic, genetic, or acquired diseases. Approximately 80% manifest as sCJD, which occurs globally at an annual rate of roughly one to two cases per million population. The phenotypic heterogeneity of sCJD presents considerable challenges for the identification of new subtypes (strains). A sporadic form of fatal insomnia, referred to as sFI, with strain properties distinct from sCJD, has also been described [108]. Approximately 10–20% of human prion diseases, including familial CJD, GSS, and fatal familial insomnia (FFI), are autosomal dominantly inherited and are associated with coding mutations in *PRNP*. Inherited human prion diseases can be transmitted to experimental animals. Acquired forms of human prion disease include Kuru, iatrogenic CJD, and vCJD.

The appearance of CJD cases in teenagers and young adults in the United Kingdom during the mid-1990s prompted considerable concern that they might be acquired by exposure to BSE. By March 1996, it became clear that the unusual clinical presentation and neuropathology was remarkably consistent in these young cases [109]. The recognition that vCJD is caused by the same prion strain as BSE [25, 110–112] raised major public health concerns. Although there was widespread population exposure to BSE, the number of vCJD cases worldwide is fortunately currently around 200. However, the uncertain effects of human genetic susceptibility and the BSE prion species barrier on incubation period make it hard to predict accurately the full extent of the vCJD epidemic. Importantly, kuru incubation times in humans can exceed 50 years in the absence of a species barrier [113]. Also

important in this regard is that a prolonged, clinically silent, incubation period increases the possibility of iatrogenic vCJD transmission. Indeed, secondary transmission of vCJD by blood transfusion does appear to be an efficient route of transmission [114, 115].

For many years the transmission of human prion diseases was studied largely with apes and monkeys where >90% of cases are thought to be transmissible [116], but the cost of housing these animals for long time periods, as well as significant ethical concerns surrounding their use, severely limited these studies. Inoculations of non-transgenic laboratory rodents produced variable results [117–121]. Generally, only ~10% of intracerebrally inoculated mice developed CNS dysfunction with incubation times of >500 days [19], limiting the ability of this approach to characterize specific agent strains.

Based on seminal studies showing that the species barrier between mice and prions from Syrian hamsters could be abrogated by expression of a hamster PrP transgene in mice [17], transgenic mice expressing human PrP were constructed. Surprisingly, two lines of Tg(HuPrP) mice expressing HuPrP with V at 129 (HuPrP-V129), referred to as Tg(HuPrP)152 and Tg(HuPrP)110, inoculated with human prions failed to develop CNS dysfunction more frequently than non-transgenic controls [19]. Subsequently, mice expressing a chimeric human/mouse PrP transgene, designated MHu2M, were constructed, because earlier studies had shown that a chimeric hamster/mouse PrP gene supported transmission of either mouse or hamster prions [11, 122]. These Tg(MHu2M)5378 mice were found to be highly susceptible to human prions, suggesting that Tg(HuPrP) mice have considerable difficulty converting $HuPrP^{C}$ into PrP^{Sc} [19]. However, Tg(HuPrP) 152 mice, and another line designated Tg(HuPrP)440, which expresses HuPrP with M at 129, when crossed with $Prnp^{0/0}$ [18], were rendered susceptible to human prions [20]. These observations demonstrated that Tg(HuPrP) mice were resistant to human prions because $MoPrP^{C}$ inhibited the conversion of $HuPrP^{C}$ into PrP^{Sc}. In contrast, Tg(MHu2M)5378 mice crossed onto the null background were only slightly more susceptible to human prions compared to Tg(MHu2M) 5378 mice that expressed both chimeric and $MoPrP^{C}$. Furthermore, Tg(MHu2M) mice inoculated with either Hu or chimeric MHu2M prions exhibited similar incubation times.

The availability of susceptible transgenic mice made possible the rapid and relatively inexpensive transmission of human prion diseases for the first time. Several additional similar transgenic mouse models have also been produced, with identical results [28, 63, 64].

Early transmission studies to Tg(MHu2M)5378 mice provided evidence that different human prion strains, in this case in patients with FFI, caused by mutation at codon 178 (D178N), and familial CJD, caused by mutation at codon 200 (E200K), are represented by different conformational states of PrP^{Sc} [123], a concept first elaborated following transmission of different hamster-adapted strains of TME [124]. While this hypothesis is supported by considerable additional experimental evidence, defining human prion strain prevalence has been hampered by difficulties in arriving at an internationally accepted classification system for

human prion strains [125, 126], and by the observation that multiple PrP^{Sc} subtypes coexist in the same brain [127]. Korth and co-workers refined the MHu2M PrP approach, and optimized human prion transmission by replacing key human PrP residues with the equivalent residues from mouse. The resulting chimera, referred to as Tg(MHu2M,M165V,E167Q) mice resulted in shortening the incubation time to approximately 110 days for prions from sCJD patients and divergence into two strain types [61]. Even shorter incubation times and CJD strain evolution were also observed in another line, termed Tg1014, in which a single additional residue (M111V) was reverted to mouse [128]. Recently, gene-targeting approaches have also been employed to produce mice expressing human PrP [129] and transmission of a limited number of sCJD cases in these mice has provided evidence for four distinct prion strains. Evidence of strain variation in sCJD have also come from laboratory studies in bank voles [130].

As previously described for the animal prion diseases, human prion disease susceptibility is strongly influenced by polymorphic variation of *PRNP*. In particular, homozygosity at *PRNP* codon 129, which encodes M or V, predisposes to the development of sporadic and acquired CJD [15, 16]. Strikingly, all neuropathologically confirmed vCJD cases studied so far have been homozygous for M at codon 129 [131]. Transmission studies of human CJD cases to transgenic mice confirm the influence of this polymorphism. While mice expressing V129 are susceptible to all PrP^{Sc} types and PrP 129 genotypes [20, 61, 111, 112, 132], mice expressing the HuPrP-129M allele are susceptible to prions from M129 homozygous patients, transmissions from patients mismatched at this codon, or heterozygotes are generally more inefficient [20, 28, 61].

Although initial BSE transmissions to Tg(HuPrP)152 mice were uniformly negative, suggesting a substantial species barrier in humans to BSE prions [132], subsequent BSE transmission to transgenic mice expressing M at human PrP codon 129 revealed a very low transmission efficiency with low attack rate and long incubation times. Moreover, a strain shift was occasionally observed in these transmissions, producing a sCJD-like phenotype in a proportion of mice [27, 28]. In contrast, gene-targeted transgenic mice expressing human PrP were not susceptible to BSE prions [27]. However, these mice were susceptible to sheep-adapted BSE prions suggesting increased susceptibility of humans to BSE prions following passage through sheep [133].

7 Conclusions and Future Perspectives

Prions share the ability to propagate strain information with nucleic acid based pathogens, but it is unclear how they mutate and acquire fitness in the absence of this informational component. Because prion diseases occur as epidemics, understanding this mechanism is of paramount importance for implementing control strategies to limit their spread, and for evaluating their zoonotic potential. While the existence and mutability of strains persuaded early researchers that prion

diseases were caused by viruses, albeit ones with unconventional properties, it is now clear to most investigators that prions encipher strain information in the conformation of PrP^{Sc} [61, 123, 124, 134, 135]. Mutational events in agent-associated nucleic acid were originally cited as the cause of strain instability [34]; however, more recently changes in the conformation of PrP^{Sc} were shown to be associated with the acquisition of new strain properties [134]. To account for the phenomena of prion transmission barriers, strain instability, heterogeneity, and adaptation in the context of PrP^{Sc} conformation, the conformational selection model postulates that only a subset of PrP^{Sc} conformations is compatible with each individual PrP primary structure [136, 137]. Recently, cell culture based systems have been developed in which prion mutation and selection can be studied [138].

Clearly, the identification of distinct strains in prion infected sheep, cervids, and cattle is of importance when considering the potential for interspecies transmission, including humans. The demonstration that disease occurred in an animal expressing a mutant form of bovine PrP, corresponding to the most common familial CJD mutation, raises important questions about the possible origins and pathogenesis of BSE and other prion diseases. Atypical scrapie, a recently-recognized and surprisingly prevalent prion disease of sheep, appears to represent another example of an emerging prion disease, of uncertain origin and species tropism. The appearance of vCJD following human exposure to BSE [110, 112], our demonstration of CWD prions in muscle [139] and antler velvet [70], as well as Race and colleagues' description of CWD prions in deer fat [140], place the human species barrier to CWD at the forefront of public health concerns. The substantial market for elk antler velvet in traditional Asian medicine, and the demonstration of CWD infectivity in this material, also warrants concern [70]. North American hunters harvest thousands of deer and elk each year. Since it is not currently mandatory to have these animals tested for CWD, it is likely that humans consume CWD prions. While CWD prions have hitherto reassuringly failed to induce disease in transgenic mice expressing human PrP [72, 75, 98], systematically addressing the zoonotic potential, as well as the tissue distributions of CWD1 and CWD2 strains in infected deer and elk, would nonetheless appear to be high priorities. Investigating the susceptibility of transgenic mice to prions strains from various species will continue to be an approach for addressing the possible origins of prion diseases as well as a sensitive means of addressing the potential for interspecies prion transmission.

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Neuroprotective and Neurotoxic Signaling by the Prion Protein

Ulrike K. Resenberger, Konstanze F. Winklhofer, and Jörg Tatzelt

Abstract Prion diseases in humans and animals are characterized by progressive neurodegeneration and the formation of infectious particles called prions. Both features are intimately linked to a conformational transition of the cellular prion protein (PrP^C) into aberrantly folded conformers with neurotoxic and self-replicating activities. Interestingly, there is increasing evidence that the infectious and neurotoxic properties of PrP conformers are not necessarily coupled. Transgenic mouse models revealed that some PrP mutants interfere with neuronal function in the absence of infectious prions. Vice versa, propagation of prions can occur without causing neurotoxicity. Consequently, it appears plausible that two partially independent pathways exist, one pathway leading to the propagation of infectious prions and another one that mediates neurotoxic signaling. In this review we will summarize current knowledge of neurotoxic PrP conformers and discuss the role of PrP^C as a mediator of both stress-protective and neurotoxic signaling cascades.

Keywords Alzheimer · Intrinsically disordered · Misfolding · Neurodegeneration · Prion

Contents

U.K. Resenberger
Neurobiochemistry, Adolf-Butenandt-Institute, Ludwig-Maximilians-University Munich, Germany

K.F. Winklhofer, and J. Tatzelt (✉)
Neurobiochemistry, Adolf-Butenandt-Institute, Ludwig-Maximilians-University Munich, Germany and
DZNE - German Center for Neurodegenerative Diseases, Munich, Germany,
e-mail: Joerg.Tatzelt@med.uni-muenchen.de

1 Prion Diseases in Humans and Animals

"*Der Trab ist auch eine Krankheit der Schaafe, und ist ansteckend. Sie schleppen sich lange, verzehren sich nach und nach, und zuletzt müssen sie sterben.*" These sentences are taken from an article published in 1759 [1] and describe two hallmarks of prion diseases or transmissible spongiform encephalopathies (TSEs, summarized in Table 1): The formation and transmission of an infectious particle and the invariably fatal course of these diseases. More than 200 years later a landmark discovery paved the way to study the pathogenesis of prion diseases at a molecular level. Prusiner and colleagues reported the identification of a protease-resistant protein in brain extracts, which co-purified with the infectious scrapie agent [18]. After the N-terminal amino acid sequence of the proteinase K (PK)-resistant core of the prion protein (PrP 27–30) was published in 1984 [19], two

Table 1 Prion diseases in humans and animals

TSE disease	Host species	Origin	First descriptions
Scrapie	Sheep, goat	Infectious	1732 [2]
Creutzfeldt–Jacob disease (CJD)	Human	Sporadic	1920 [3]
		Familial	1921 [4]
		Infectious	
		Iatrogenic	
Gerstmann–Sträussler–Schenker Syndrome (GSS)	Human	Familial	1936 [5]
Transmissible mink encephalopathy (TME)	Mink	Infectious	1947 [6]
Kuru	Human	Infectious	1957 [7]
			1959 [8]
			1966 [9]
Chronic wasting disease (CWD)	Cervid	Infectious	1978 [10]
Fatal familial insomnia (FFI)	Human	Familial	1986 [11]
Bovine spongiform encephalopathy (BSE)	Cattle	Infectious	1987 [12]
Exotic ungulate encephalopathy (EUE)	Ungulate	Infectious	1988 [13]
			1990 [14]
Feline spongiform encephalopathy (FSE)	Feline	Infectious	1991 [15]
Sporadic fatal insomnia (SFI)	Human	Sporadic	1999 [16, 17]

independent groups identified the PrP gene (Prnp) [20, 21]. Remarkably, the prion protein was found to be encoded by a host gene, which is constitutively transcribed under physiological conditions. From these and subsequent studies it emerged that it is not the expression of the prion protein that is the disease-specific feature, but rather its biophysical and biochemical properties (reviewed in [22–25]). In contrast to the cellular prion protein PrP^{C}, the disease-specific scrapie-prion protein (PrP^{Sc}) is insoluble in non-ionic detergents, partially resistant to proteolytic digestion, and characterized by a high content of β-sheet secondary structure [26–28]. While the structure of PrP^{C} has been determined by NMR at high resolution [29–31], no such information is available for PrP^{Sc}.

The central role of PrP^{C} in the pathogenesis of prion diseases was demonstrated in transgenic mice with a targeted disruption of the PrP gene: $PrP^{0/0}$ mice are resistant to prion diseases and do not propagate infectious prions [32]. Moreover, inherited prion diseases in humans are causally linked to mutations in Prnp (reviewed in [22, 33]).

Another interesting feature of prion diseases is their zoonotic potential. The first indication that prions can be transmitted to heterologous species was the outbreak of a prion disease in farmed mink in 1947, which was probably introduced by scrapie-infected animal feed [6]. Experimental transmission of Kuru to chimpanzees further supported the notion that prion diseases can be transmitted among different species [9]. The fact that animal prion diseases can be a major threat to public health became apparent only after the onset of variant CJD (vCJD), a human prion disease originating from consumption of food products contaminated with bovine spongiform encephalopathy (BSE) prions [12]. While BSE has largely been confined, another prion disease in animals might be of interest in this context. Initially recognized in domestic Colorado mule deer [10], chronic wasting disease (CWD) affects free ranging deer and is endemic in large areas in North America. For details on CWD and its possible impact on public health, please see the review by Hermann Schätzl [34].

2 Aberrant Folding of PrP, Infectious Prions, and Neurodegeneration

Like other neurodegenerative disorders, such as Alzheimer's and Parkinson's disease, prion diseases are characterized by the formation and accumulation of an aberrantly folded protein in the brain. However, a unique feature of prion diseases is their transmissibility. Prions (acronym for proteinaceous infectious particles) are mainly composed of PrP^{Sc}, polysaccharides, and lipids, but lack nucleic acids longer than 25 nucleotides, arguing against an essential role for DNA/RNA in mediating infectivity [35, 36]. Indeed, recent experiments support the protein-only hypothesis: recombinant PrP expressed in and purified from bacteria and subsequently misfolded in vitro can transmit the disease [37–40].

Further evidence for the phenomenon of self-propagating protein conformers was provided by the discovery of proteins with prion-like properties in the budding yeast *Saccharomyces cerevisiae* [41]. The chapter by Mick Tuite [165, 166] in this book will provide a detailed review about fungal prions and their cellular functions. In addition, it has been reported that the translation regulator CPEB from *Aplysia* can act as a self-sustaining prion-like protein in the nervous system [42–44].

In the majority of prion diseases there is a correlation between the accumulation of PrP^{Sc}, formation of infectious prions, and neurodegeneration. Indeed, the diagnosis of prion diseases is mainly based on the detection of PK-resistant PrP. However, transgenic mouse models revealed that several PrP mutants can induce neuronal cell death in the absence of infectious prion propagation and accumulation of PK-resistant PrP [45–52]. In contrast, it has been shown that propagation of infectious prions can occur in the absence of clinical signs [53–55]. Consequently, it appears plausible that two partially independent pathways exist, one pathway leading to the propagation of infectious prions and another that mediates neurotoxic signaling [56].

In the remaining part of this review we will concentrate on the neurotoxic activity of aberrant PrP conformers and the neuroprotective capacity of the natively folded cellular prion protein PrP^{C}. Interestingly, both activities seem to be interconnected.

3 PrP Mutants Can Induce Neurodegeneration in the Absence of Prion Propagation

A major challenge in neurodegenerative disease research is to understand how the formation of β-sheet-rich protein assemblies, regardless of whether they are formed by PrP, Aβ, tau, polyQ-containing proteins, or α-synuclein, are causally linked to neuronal dysfunction. As noted above, several PrP mutants have a highly neurotoxic potential in the absence of infectious prion replication. In the following paragraphs we briefly introduce these neurotoxic PrP mutants, which allow the generation of valuable cellular and animal models to decipher toxic pathways induced by aberrantly folded protein species in neuronal cells.

3.1 ^{Ctm}PrP

Mature PrP^{C} is modified with two N-linked glycans of complex structure [57, 58] and tethered to the outer leaflet of the plasma membrane via a C-terminal glycosylphosphatidylinositol (GPI) anchor [59]. Interestingly, during translocation into the endoplasmic reticulum (ER), two transmembrane forms of PrP can be formed [60]. The different topologies are termed ^{Ntm}PrP (N-terminus facing the ER lumen) and ^{Ctm}PrP (C-terminus facing the ER lumen) with the internal hydrophobic

domain (HD, amino acids 112–135) serving as a putative transmembrane domain. Mutations within the HD or the N-terminal signal peptide were found to alter the relative amount of ^{Ctm}PrP and ^{Ntm}PrP [46, 61, 62]. Increased synthesis of ^{Ctm}PrP has been shown to coincide with progressive neurodegeneration both in Gerstmann–Sträussler–Scheinker syndrome (GSS) patients with an A117V mutation and in transgenic mice carrying a triple mutation within the HD (AAA113,115,118VVV, in short AV3) [46, 63]. The accumulation of PrP^{Sc} has been reported to enhance the generation of ^{Ctm}PrP [64], but this concept was challenged by a different study [65]. How could ^{Ctm}PrP induce neurodegeneration? A transgenic mouse model revealed that neurotoxicity of ^{Ctm}PrP is strongly dependent on the co-expression of wild-type PrP^{C} [63]. This observation could indicate that an interaction between ^{Ctm}PrP and PrP^{C} "abuses" a PrP^{C}-dependent signaling pathway for toxic signaling, similarly to PrP^{Sc} (see below) or a PrP mutant which lacks the HD (PrPΔHD) (reviewed in [66]). In another study it was shown that ^{Ctm}PrP can interact with and disrupt the function of Mahogunin, a cytosolic ubiquitin ligase whose loss of function causes spongiform neurodegeneration in mice [67]. Whether this pathway can also be modulated by wild-type PrP^{C} is not yet known.

3.2 cytoPrP

A transgenic mouse model expressing a PrP mutant without the N-terminal signal sequence (cytoPrP) conclusively showed that targeting of PrP to the cytosolic compartment can be neurotoxic [49, 68]. Further evidence for a toxic potential of cytosolically localized PrP was provided by additional mouse models [67, 69], by several mammalian cell culture models [49, 70, 71], and by a yeast model [72].

The presence of secretory proteins in the cytosolic compartment is normally regarded as a consequence of a quality control pathway designated ER-associated degradation (ERAD), which mediates post-translational degradation of non-native proteins generated in the endoplasmic reticulum. (reviewed in [73–75]). Based on our studies in yeast and mammalian cells, we propose that an impaired import into the ER rather than retrograde translocation from the ER is responsible for the occurrence of cytosolic PrP [72, 76–78]. In support of the notion that PrP is targeted to the cytosol via a co-translocational pathway, cytosolic PrP is characterized by the presence of an uncleaved N-terminal ER signal peptide [71, 76, 78, 79]. Processing of the N-terminal signal peptide and the transfer of core glycans occur co-translocationally prior to the release of the polypeptide into the ER lumen (reviewed in [80]). Thus, an uncleaved signal peptide is indicative of an abrogated translocation event.

How could the translocation efficiency of PrP into the ER be regulated? The most important factor clearly is the N-terminal signal sequence (reviewed in [81]), but, in addition, two other mechanisms have been described to favor the formation of cytosolically localized PrP. By alternative initiation of translation from a downstream AUG, an N-terminally truncated PrP can be generated, which

localizes to the cytosol and impairs cell viability [82, 83]. In addition, we could recently demonstrate that intrinsically disordered domains, such as the N-terminal domain of PrP, can modulate translocation efficiency and target secretory proteins to a co-translocational quality control pathway [78, 84]. Consistent with the idea that an imbalance of cellular homeostasis brings a co-translocational quality control pathway to the scene [85], the accumulation of cytosolic PrP containing an uncleaved signal peptide was favored under conditions of ER stress [86, 87].

Different mechanisms have been described to explain the toxic effects of PrP in the cytosolic compartment. In one study co-aggregation of misfolded cytoPrP with the anti-apoptotic protein Bcl-2 was shown to coincide with toxicity [71]. Another study reported that cytoPrP can interact with the E3 ubiquitin ligase Mahogunin, thereby disrupting its function [67]. Interestingly, a toxic potential was also observed for cytosolically localized PrP^{Sc}, which can inhibit proteasomal activity [69].

3.3 PG14-PrP

While the majority of pathogenic mutations are found in the structured C-terminal domain of PrP, a nine octarepeat insertion (PG14) in the intrinsically disordered N-terminal domain is also associated with an inherited prion disease in humans [88] (for a detailed review on the genetics of prion disease see [33]). Expression of PG14-PrP in transgenic mice resulted in a slowly progressive neurological disorder characterized clinically by ataxia. Neuropathologically, PG14-PrP transgenic mice displayed cerebellar atrophy and granule cell loss, gliosis, and deposition of weakly PK-resistant PrP aggregates. Remarkably, brain extracts from PG14-PrP transgenic mice did not transmit the disease [45, 89]. However, after inoculation of mouse prions (RML strain), PG14-PrP transgenic mice developed typical symptoms of murine scrapie and propagated highly protease-resistant PrP and infectious prions [89]. Another study revealed that the infectious PrP aggregates formed in RML-infected PG14-PrP transgenic mice and the non-infectious PrP aggregates found in the brain of uninfected PG14-PrP transgenic mice have multiple biochemical and structural features in common [90]. PG14-PrP can form intra-axonal aggregates that disrupt axonal transport [91] and, compared to wild-type PrP^{C} PG14-PrP, has a reduced neuroprotective activity [92]. Thus, the PG14-PrP mutant supports the concept that the adverse effects of protein misfolding in neurodegeneration can be a combination of both a gain of toxic function and a loss of physiological function (reviewed in [93]).

3.4 PrPΔHD

From a study designed to identify regions of PrP^{C} involved in the formation of PrP^{Sc}, it emerged that deletions in the internal hydrophobic domain (HD) can

convert PrP into a neurotoxic protein [48, 51, 52]. Notably, the deletion of 20 amino acids of the HD (Δ105–125) are sufficient to create a neurotoxic mutant denoted PrPΔCR [52]. Transgenic mice expressing PrPΔHD mutants developed a spontaneous neurodegenerative disease in the absence of infectious prion propagation. Surprisingly, the neurotoxic activity of PrPΔHD mutants could be completely rescued by the co-expression of wild-type PrP^C. In this context it is important to note that deletion of the HD seems not to interfere with the maturation and cellular trafficking of PrP. Similarly to wild-type PrP^C, PrPΔHD is complex glycosylated and linked to the plasma membrane via a GPI anchor [94]. Apoptotic cell death induced by PrPΔHD and the protective activity of co-expressed PrP^C have also been demonstrated in cultured cells [95, 96]. In addition, PrPΔCR expression in cultured cells has been shown to induce spontaneous ionic currents, which could be silenced by the overexpression of wild-type PrP^C [97], and to sensitize cells to the toxic effects of aminoglycosides and bleomycin analogs [98].

Different models have been proposed to explain the toxic signaling induced by PrPΔHD. A common denominator of these models is the existence of a co-receptor at the plasma membrane, which mediates stress-protective signaling by PrP^C (see below). In the absence of PrP^C, PrPΔHD could abuse this co-receptor for neurotoxic signaling [48, 51, 52, 95].

4 Physiological Function of PrP^C

So far we have focused on the pathological activities of aberrant PrP conformers which have the capacity to induce neurodegeneration and/or to form infectious particles. Another important question yet to be addressed is what might be the physiological function of PrP^C? Importantly, PrP^C is a highly conserved protein that is found in all tetrapods and in birds [99]. Moreover, the structure of PrP^C from different species is highly similar [100], suggesting that the function of PrP^C is evolutionary conserved.

The most prominent phenotype of PrP^C-deficient mice is their resistance to prion infection, but no other striking phenotype was initially observed [101, 102]. Therefore, it has been suggested that the loss of PrP^C function may be compensated by other proteins. A closer analysis of $PrP^{0/0}$ mice revealed several subtle phenotypic alterations, such as altered circadian activity rhythms and patterns [103], modified responses to oxidative stress due to a reduced activity of Cu/Zn superoxide dismutase [104], functional changes in the glutamatergic system [105, 106], peripheral chronic demyelinating polyneuropathy [107], reduced levels of anxiety [108], age-related motor behavioral and neuropathological deficits [109, 110], retarded regeneration of adult muscle [111], and alterations in synaptic function [112]. Based on these findings, several physiological roles have been attributed to PrP^C, such as modulation of synaptic transmission and neuronal excitability, protection against oxidative stress, and a role in cell differentiation and neuronal adhesion (reviewed in [113, 114]).

These findings strongly support the notion that physiologically active PrP^C is part of signaling cascades implicated in various cellular processes. Of note, GPI-anchored proteins like PrP^C do not have direct contact with the cytosolic compartment and therefore require co-factors for intracellular signal transmission (reviewed in [115]). Attractive candidates for cellular components involved in PrP^C-dependent signaling include NMDA receptors [106] and the intracellular tyrosine kinase Fyn [116, 117].

5 Role of PrP^C as a Stress-Protective Protein

5.1 *PrP^C Protects Against Neurotoxic Stressors*

Evidence for a stress-protective activity of PrP^C was first provided by experiments with primary neurons from $PrP^{0/0}$ mice [118]. Using murine stroke models it was then demonstrated that PrP^C confers neuroprotection after an ischemic insult [119–123]. These findings in transgenic animals were corroborated and complemented by several studies in cultured cells, demonstrating a protective effect of PrP^C against neuronal cell death induced by oxidative stress [124, 125], copper [126], protein synthesis inhibitors [127, 128], and excitotoxins [95, 96] (Fig. 1).

In search for PrP-interacting proteins, murine stress-inducible protein 1 (STI1) was identified as a cell surface ligand for PrP^C. Moreover, an interaction of STI1 with PrP was implicated in the activity of PrP^C to mediate neuronal survival and differentiation [127–133]. STI1 and its human homolog Hop (Hsp70/Hsp90-organizing protein) has initially been identified as a co-chaperone of Hsp70 and Hsp90 and is predominately localized in the cytoplasm [134]. Subsequently, it was shown that STI1 may also be present in the nucleus [128, 135, 136], at the plasma membrane [128], and can even be secreted by astrocytes [137].

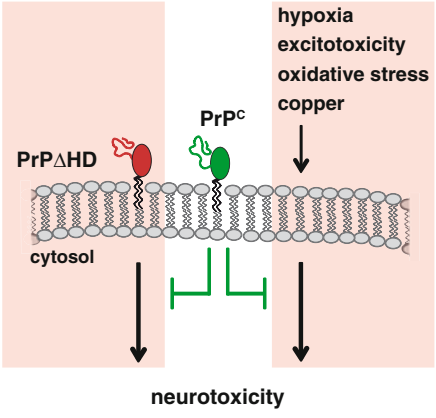

Fig. 1 Stress-protective activity of PrP^C. PrP^C mediates protection from various neurotoxic stimuli, such as hypoxia, excitotoxicity, oxidative stress, and expression of a neurotoxic PrP mutant lacking the hydrophobic domain (PrPΔHD)

We recently employed a cell culture model to show that PrP^C can protect from cell death induced by excitotoxins, such as glutamate or kainate [95, 96]. With the help of this model, we could identify domains of PrP^C required for this stress-protective activity. Corroborating previous findings in mice [123], the stress-protective activity of PrP^C was dependent on the intrinsically disordered N-terminal domain [95, 96]. In addition, we identified two additional domains essential for the protective activity of PrP: the internal hydrophobic domain (HD) and the C-terminal GPI anchor. In contrast to the highly structured C-terminal domain, the N-terminal domain of PrP^C is intrinsically disordered [29–31]. Intrinsically disordered domains have been shown to mediate protein–protein interactions [138]. Thus, it is conceivable that the N-terminal domain of PrP^C is involved in the interaction with a yet unidentified transmembrane protein, which is required to induce signal transduction. Similarly, targeting of PrP^C to specialized microdomains at the plasma membrane by its C-terminal GPI anchor might be a prerequisite for such a specific interaction of PrP^C with signaling molecules. Our analysis of PrP^C in cultured cells and mouse brain by native PAGE and cross-linking approaches provided experimental evidence that PrP^C can form homo-dimers and that deletion of the HD interferes with dimer formation [95]. Indeed, previous in vitro studies indicated that native PrP^C purified from bovine brain exists as a monomer-dimer equilibrium [139]. Based on these and additional experiments, it has been concluded that the HD is part of a dimer interface and that dimer formation of PrP^C is essential for its neuroprotective activity [95].

5.2 *PrP^C Protects Against Toxic Effects of PrPΔHD*

Two intriguing activities of PrP emerged from studies with transgenic mice expressing mutants lacking the internal hydrophobic domain (HD). First, deleting residues 105–125 from the HD was sufficient to convert PrP from a stress-protective into a neurotoxic protein [52]. Second, co-expression of wild-type PrP^C completely blocked the toxic activities of PrPΔHD mutants [48, 51, 52] (Fig. 1). The toxic activity of PrPΔHD mutants could be related to that of Doppel, a neurotoxic protein with a tertiary structure similar to that of the C-terminal domain of PrP^C [140]. Notably, Doppel-induced neurodegeneration is also rescued by the co-expression of PrP^C [141–144]. A comprehensive review of Doppel is provided by David Westaway in this book.

Concerning the effect of PrP^C on PrPΔHD-induced toxicity, the following scenarios are conceivable. (1) PrP^C and PrPΔHD compete for a common receptor and/or ligand to which PrP^C binds with a higher affinity than does PrPΔHD. In the absence of PrP^C, the interaction between PrPΔHD and the receptor and/or ligand induces cell death. (2) PrP^C might modulate indirectly a yet unknown PrPΔHD-mediated toxic signaling pathway. The nature of the putative co-receptor of PrP^C/PrPΔHD is still enigmatic. Notably, the stress-protective signaling of

PrP^C is linked to dimer formation with the HD as part of the dimer interface [95]. Thus, failure of dimerization might be an additional factor involved in PrPΔHD-induced toxicity.

6 PrP^C as a Mediator of Neurotoxic Signaling

From the findings reviewed above it emerged that pathogenic mutations in PrP can cause a loss of the stress-protective activity of natively folded PrP^C and a gain of toxic function due to the formation of aberrantly folded PrP conformers. This concept is in line with the prevailing idea of how aberrant protein folding contributes to the pathomechanisms of neurodegenerative diseases in general, be it Alzheimer's disease, Parkinson's disease, or polyQ expansion disorders (reviewed in [93]). However, the mechanisms seem to be even more complex in prion diseases. In the remaining paragraphs we summarize evidence that physiologically active PrP^C is required to mediate toxic signaling of scrapie prions and possibly even of pathogenic protein conformers associated with other neurodegenerative diseases (Fig. 2).

6.1 GPI-Anchored PrP^C Mediates Toxic Signaling of Scrapie Prions

To study a possible role of PrP^C as a mediator of toxic signaling independently from its essential function in PrP^{Sc} propagation is experimentally demanding. An elegant approach was described by Brandner and colleagues who grafted neural tissues overexpressing PrP^C into the brains of $PrP^{0/0}$ mice. After an intracerebral inoculation of scrapie prions the PrP^C-expressing graft propagated PrP^{Sc} and developed histopathological alterations characteristic of scrapie disease, but the adjoining PrP^C-deficient tissue remained healthy despite the presence of high PrP^{Sc} levels [53]. In a different mouse model it was shown that interruption of neuronal PrP^C expression during an ongoing prion infection prevents neuronal loss and reverses early spongiform changes [55]. By antibody-induced cross-linking of PrP^C it has been demonstrated in vivo that PrP^C is able to induce neurotoxic signaling pathways [145]. After injection of PrP^C-specific monoclonal antibodies into the hippocampus of C57BL/10 mice, neuronal damage was observed in CA1, CA2, and CA3 cell body layers and in the dentate gyrus. Finally, a transgenic mouse model revealed that the GPI anchor of PrP^C is an important factor for mediating toxic signaling of PrP^{Sc}. Chesebro and colleagues generated transgenic mice expressing PrP without a C-terminal GPI anchor (PrPΔGPI) in the absence of wild-type GPI-anchored PrP^C [54]. As a consequence, PrPΔGPI is secreted from neurons, a feature already shown before in cultured cells [94]. Even though PrPΔGPI seems to be

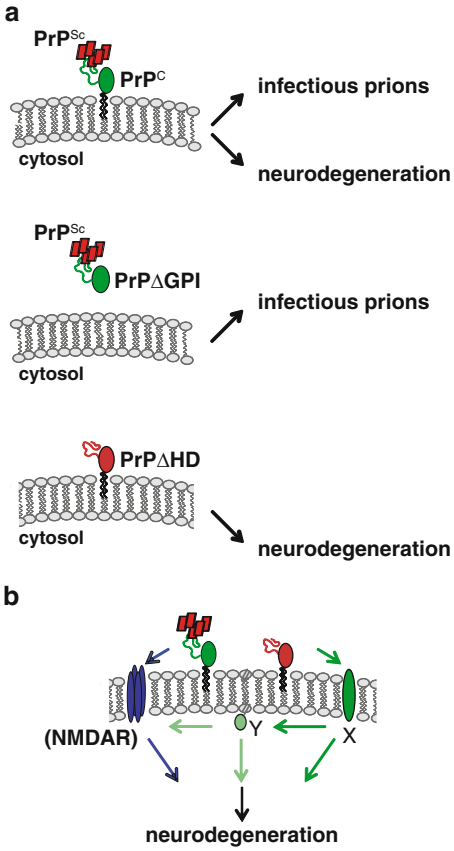

Fig. 2 Propagation of infectious prions and neurodegeneration in prion diseases. (**a**) Propagation of infectious prions and neurodegeneration can occur independently. In the majority of prion diseases there is a correlation between the accumulation of PrP^{Sc}, formation of infectious prions, and neurodegeneration, which is dependent on the expression of GPI-anchored PrP in neuronal cells (*upper panel*). Infection of transgenic mice expressing an anchorless PrP mutant (PrPΔGPI) results in the formation of infectious prions; however, the onset of clinical symptoms is at least significantly delayed (*middle panel*). Expression of a PrP mutant with a deletions in the internal hydrophobic domain (PrPΔHD) induces neuronal cell death in the absence of prion propagation (*lower panel*). (**b**) Putative model of toxic signaling induced by PrP^{Sc} or PrPΔHD at the plasma membrane. Since PrP^{C} has no direct contact to the cytosolic compartment, it is plausible to assume that intracellular signal transmission induced by PrP^{Sc} or PrPΔHD involves additional cellular factors, such as different transmembrane proteins (X), and/or cytosolic proteins associated with lipid rafts (Y). Activation of the NMDA receptor (NMDAR) could be involved either directly through an interaction with pathogenic PrP conformers or indirectly via modulation by cytosolic factors

partially misfolded, the expression of this PrP mutant in transgenic mice or cultured cells is not toxic [54, 71]. Remarkably, after inoculating transgenic mice expressing PrPΔGPI with scrapie prions, secreted PrPΔGPI sustained propagation of infection

prions, but the onset of clinical symptoms was at least significantly delayed [54]. This study also strengthened the notion that propagation of infectious prions and PrP^{Sc}-induced neuronal cell death are separate pathways.

Based on the different animal and cell culture models, two plausible scenarios for the toxic effects of PrP^{Sc} can be envisaged – either neurotoxicity of PrP^{Sc} is linked to its propagation within neuronal cells, which is dependent on the expression of PrP^{C} or PrP^{Sc} elicits a deadly signal though a PrP^{C}-dependent signaling pathway. The latter aspect was supported by a newly established cell-culture model in our group showing that PrP^{Sc}-induced cell death is dependent on the expression of GPI-anchored PrP^{C}. Of note, this model also revealed that domains required for the stress-protective signaling of PrP^{C}, i.e., the N-terminal intrinsically disordered domain and the C-terminal GPI anchor, are also required to mediate toxic signaling induced by PrP^{Sc} [95].

What might be the underlying mechanism linking the neuroprotective activity of PrP^{C} to the PrP^{C}-dependent neurotoxic effects of PrP^{Sc}? PrP^{Sc} could modulate the interaction of PrP^{C} with its putative co-receptor and/or ligand, possibly due to the formation of a PrP^{C}/PrP^{Sc} complex. As a consequence, there is a switch from stress-protective to toxic signaling, driven by a conformational change of the receptor leading to its overstimulation. Compatible with the model of a common PrP receptor for both pro-apoptotic and anti-apoptotic signaling is the toxic potential of PrP lacking the hydrophobic domain (PrPΔHD, see above).

6.2 *A Possible Role of PrP^{C} in Other Neurodegenerative Diseases*

Aberrant protein folding is a common hallmark of neurodegenerative diseases. Interestingly, misfolded conformers of different disease-associated proteins share similar biophysical and biochemical features. Consequently, it appears logical to analyze whether PrP could also be implicated in the pathogenesis of Alzheimer's disease. Alzheimer's disease is characterized by the deposition of Aβ peptides in extracellular amyloid plaques and hyperphosphorylated tau in intraneuronal neurofibrillary tangles (reviewed in [146, 147]). Indeed, it was reported that extracellular plaques in AD patients contain PrP [148, 149] and, vice versa, that plaques found in CJD patients' brains contain Aβ [150, 151]. In addition, the M129V polymorphism in the PrP gene was shown to increase the risk for early onset AD [152]. In a transgenic mouse model, PrP^{C} expression promoted Aβ plaque formation [153]. However, in a cell culture model overexpression of PrP^{C} led to a decrease in Aβ formation by inhibition of β-cleavage of the amyloid precursor protein (APP) [154].

The findings summarized above may indicate a possible link between PrP and Alzheimer's disease; however, it was quite surprising that PrP^{C} has been reported to act as a high-affinity receptor for beta-sheet-rich Aβ oligomers. The interaction of PrP^{C} with Aβ has now been demonstrated in several independent studies

[155–158], but the functional consequences of such an interaction are still controversially discussed. The observation that Aβ-induced inhibition of long-term potentiation (LTP) and memory impairment in transgenic Alzheimer mice requires PrP^C [155, 159] could not be reproduced by other groups [157, 158, 160]. In a different approach it was shown that an intraperitoneal injection of anti-PrP^C monoclonal antibodies restores cognitive deficits in an Alzheimer's disease model mouse [161]. These seemingly contradictory observations might be explained by differences in the experimental approaches employed by different groups. The most important limitation is that there is no mouse model available that faithfully replicates all major aspects of AD pathology (reviewed in [162, 163]). Different lines of transgenic mice with variable phenotypes (particularly concerning LTP) were used to prove or disprove the effects of PrP on Aβ toxicity, a fact that impedes a direct comparison of the different studies. Moreover, the toxic Aβ species are poorly defined. Recombinant Aβ conformers have to be prepared freshly and contain a mixture of unstable oligomers that are likely to undergo further conformational transition during the experimental procedure. Thus, it is impossible to compare the Aβ preparations employed by different groups. However, these studies convincingly demonstrated that PrP^C at the plasma membrane can bind to β-sheet-rich Aβ conformers. While the functional relevance of an interaction between PrP^C and Aβ is still unclear, it would be rather surprising that such an interaction occurs without any consequences. Indeed, a study from our group suggests that PrP^C has an intrinsic ability to interact with β-sheet-rich (β)-conformers independent of their primary sequence [164]. Employing established cell lines and primary neurons prepared from wild-type or $PrP^{0/0}$ mice we could show that expression of PrP^C sensitizes cells to toxic effects of various β-conformers of completely different origins, formed by (1) heterologous PrP, (2) amyloid-β peptide, (3) yeast prion proteins, or (4) designed β-peptides. By making use of different PrP mutants, we were able to show that the C-terminal GPI anchor and the intrinsically disordered N-terminus are important domains for toxic signal transduction via PrP^C. Notably, the N-terminal domain mediated binding to the β-conformers, and a secreted version of the isolated N-terminal domain interfered with toxic signaling via PrP^C [164]. The next challenge will be the identification of cellular factors and pathways involved in PrP^C-mediated signaling.

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Prion Seeded Conversion and Amplification Assays

Christina D. Orrú and Byron Caughey

Abstract The conversion of the normal prion protein (PrP^C) into its misfolded, aggregation-prone and infectious (prion) isoform is central to the progression of transmissible spongiform encephalopathies (TSEs) or prion diseases. Since the initial development of a cell free PrP conversion reaction, striking progress has been made in the development of much more continuous prion-induced conversion and amplification reactions. These studies have provided major insights into the molecular underpinnings of prion propagation and enabled the development of ultra-sensitive tests for prions and prion disease diagnosis. This chapter will provide an overview of such reactions and the practical and fundamental consequences of their development.

Keywords Prion composition · PMCA · QuIC · ASA · Transmissible · Spongiform · Ecephalopathies

Contents

C.D. Orrú and B. Caughey (✉)
Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT 59840, USA
e-mail: bcaughey@nih.gov

1 Introduction

A major issue in coping with any infectious disease is the ability to detect the responsible pathogen. In the case of the transmissible spongiform encephalopathies (TSEs) or prion diseases of mammals, it is increasingly apparent that the pathogen is a misfolded multimeric form of the host's prion protein (PrP) [1]. This infectious protein, PrP^{Sc}, can instigate its own propagation by binding to its normal counterpart, PrP^{C} or PrP^{sen}, and inducing its conversion to a form that tends to be higher in beta sheet content, polymeric, and more protease-resistant. The lack of an agent-specific nucleic acid genome negates the possibility of ultrasensitive detection of prions by nucleic acid amplification methods such as PCR. The fact that the infectious agent is mainly comprised of a host protein also restricts the use of antibody-based detection methods to those based on conformational epitopes or epitope exposure. However, the apparent seeded/templated conformational conversion mechanism of prion propagation can be exploited to detect the presence of prions. Here we summarize recent developments in the characterization and detection of prions using assays based on seeded conversion of PrP^{C}.

2 Cell-Free Conversion Assays

The ability of PrP^{res} to induce the conversion of PrP^{C} to PrP^{res} was initially demonstrated in cell-free reactions in which brain-derived PrP^{res} was incubated with radioactively labeled PrP^{C}, which, under suitable conditions, bound to the PrP^{res} and became similarly partially protease-resistant [2]. These first generation cell-free conversion (CFC) reactions were shown to be highly specific in ways that correlated with prion transmission barriers [3–7] and strains [8]. However, the newly generated PrP^{res} was usually substoichiometric relative to the initial PrP^{res} seed, and was not demonstrably associated with new infectivity [9]. As a result, these CFC reactions (reviewed in [10]) were not suitable for sensitive detection of PrP^{res} or prions.

3 Protein-Misfolding Cyclic Amplification and Recombinant Protein-Misfolding Cyclic Amplification

In 2001 Soto and colleagues described a new type of cell-free prion conversion reaction called protein-misfolding cyclic amplification (PMCA) which has greatly improved efficiency, continuity, and sensitivity compared to the initial CFC reactions [11]. In the typical PMCA reaction, crude brain extracts are used as a source of the PrP^{C} which is induced to convert by prions or PrP^{res} in the test sample. Under these conditions, PrP^{res} can be amplified to levels that are detectable

by immunoblotting. Prion amplification by PMCA involves repeated cycles of incubation and sonication during which growing multimers of PrP^{res} are fragmented by sonication to increase the effective seed concentration. Sensitivity can also be increased by performing serial rounds of PMCA by transferring a small proportion of one reaction into fresh PrP^{C} substrate for the subsequent round. Originally developed using the hamster adapted 263K scrapie strain, the PMCA has now been adapted to many other species [12] such as sheep [13], deer [14], mice [15], and humans [16, 17]. Also, Pastrana and colleagues showed the ability of the PMCA to detect relatively PK sensitive forms of PrP^{Sc} (i.e., $sPrP^{Sc}$) [18].

PMCA is capable of extremely sensitive detection of PrP^{res} in tissues, including hamster and mouse blood [19–22] or environmental samples such as water [23]. To improve the assay's practicality an automated microplate horn system dubbed serial automated PMCA (saPMCA) was developed [21, 24]. This system allows the detection of as little as 1.2 ag or ~26 molecules of PrP^{res} after seven reaction rounds. The ability of the PMCA to detect miniscule amounts of PrP^{res} and its applicability to human CJD, sheep scrapie, and deer CWD support its use as a TSE pre-clinical diagnostic test. Recently, Chen et al. have reported a quantitative PMCA (qPMCA) approach that allows the determination of the concentration of small amounts of prions in biological samples [25]. This quantitation strategy is based on the direct correlation between the amount of PrP^{Sc} in a given sample and the number of PMCA rounds necessary to detect it. However, limitations of the assay for routine clinical applications include the need for (1) brain extracts as a source of PrP^{C} substrate, (2) multiple reaction rounds over several days to 3 weeks to achieve the best sensitivity, and (3) PK digestion and immunoblotting of PMCA products which would impede high-throughput applications.

To improve the speed and practicality of the PMCA, Atarashi et al. used recombinant PrP^{C} ($rPrP^{C}$) as a substrate instead of brain derived PrP^{C} [26]. $rPrP^{C}$ has the advantage of being easily manipulated genetically, purified in large quantities, and added to PMCA reactions at concentrations sufficient to accelerate conversion. The resulting reaction, designated rPrP-PMCA or, more briefly, recombinant Protein-Misfolding Cyclic Amplification (rPMCA) (Fig. 1), was shown initially to be able to detect as little as 50 ag of PrP^{res} and to differentiate between scrapie-infected and uninfected hamsters using 2 μL of cerebral spinal fluid (CSF).

A ground-breaking consequence of being able to propagate PrP^{res} in various cell-free reactions was the opportunity to evaluate directly the protein-only hypothesis for TSE prions. Initial indications that synthetic recombinant PrP amyloid fibrils alone can be infectious was reported by Legname and colleagues, who showed that such fibrils could induce or accelerate transmissible neurodegenerative disease in transgenic mice (Tg9944) that overexpressed the same truncated PrP mutant that was used to make the fibrils [27]. However the rPrP fibrils were non-infectious for wild-type mice, indicating that infectivity titers were extremely low. Moreover, the initial report left open the possibility that prions were being generated spontaneously in the Tg9944 mice, but evidence to the contrary has recently been published [28].

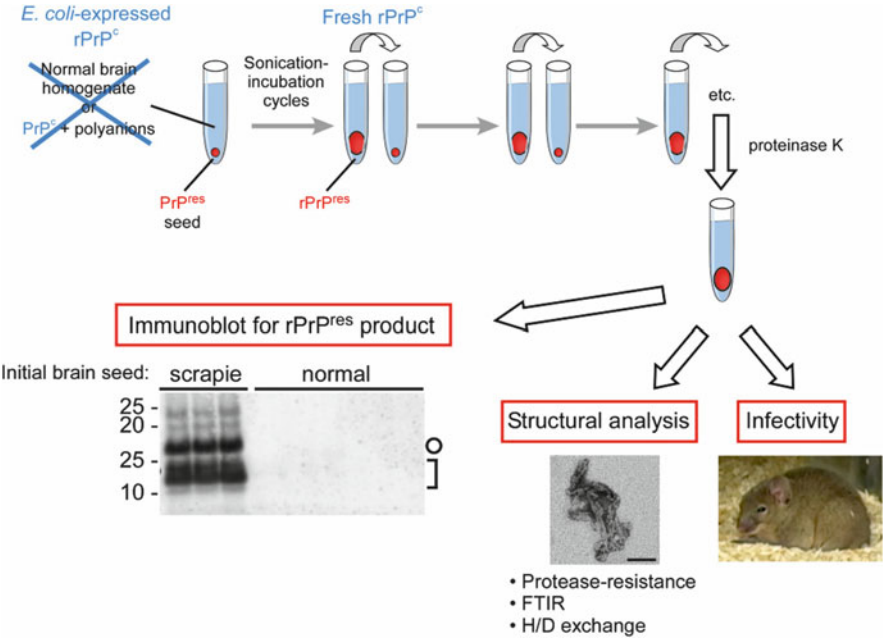

Fig. 1 Summary of the biochemical, structural and biological studies on recombinant-PMCA (r-PMCA) products. Immunoblot and electron microscopy image adapted from [26]

Much more robust TSE infectivity for wild-type rodents has since been propagated in brain homogenate-based PMCA reactions by Castilla, Soto, and colleagues [29]. Their study showed evidence that the biochemical, structural and biological characteristics of PMCA-propagated PrP^{res} was almost indistinguishable from PrP^{res} produced in vivo, except for the observation of substantially longer incubation periods obtained upon inoculation of the former into animals.

Weber and colleagues then focused on the cause of the prolonged incubation periods obtained with sPMCA-generated PrP^{res} [30–32]. They described a sonication-dependent reduction in PMCA-generated PrP^{res} aggregate size and suggested that enhanced clearance of such aggregates might explain the longer incubation periods. This interpretation is consistent with their ability to shorten the incubation periods by adsorbing the sPMCA PrP^{res} products to nitrocellulose particles prior to inoculation.

Green and colleagues demonstrated PMCA amplification of naturally occurring CWD infectivity [33]. They reported an equal level of infectivity being present in both the CWD PMCA conversion product and the original 04–22412 CWD cervid brain homogenate inoculum.

Studies by Deleault, Supattapone and colleagues demonstrated that infectious PrP^{res} propagation can be achieved in a greatly simplified system containing largely purified PrP^{C} from brain and co-purified lipids [34]. Moreover, as previously reported by the same group using another in vitro PrP^{Sc} amplification assay [35],

the PMCA's amplification efficiency was improved when carried out in the presence of accessory polyanions, i.e., single stranded synthetic poly A RNA. In the presence of such RNA molecules, infectious PrP^{res} was even generated spontaneously, i.e., without seeding with brain-derived PrP^{Sc}. The PMCA-propagated material had a titer of $\approx 5 \times 10^3$ LD_{50} per mL when intracerebrally (i.c.) inoculated into wild type hamsters. Mechanistic studies have described the selective integration of poly A RNA molecules into stable complexes with PrP molecules in the process of prion formation in vitro, suggesting that even in the absence of PrP^{Sc}, polyanionic molecules can induce a molecular reorganization of purified PrP^{C}, resulting in a conformation similar to that of PrP^{Sc} [36].

More recently, Wang and colleagues [37] described the generation of a recombinant prion with features typical of in vivo-generated prions using three components: $rPrP^{C}$, POPG (1-palmitoyl-2-oleoylphosphatidylglycerol), and RNA. Following intracerebral inoculation in wild type mice the clinical stage of disease was reached at ~150 days. Biochemical characterizations of the PrP^{res} generated, as well as the clinical symptoms, histopathology, and second passage behavior induced by its inoculation strongly supported the conclusion that they had generated TSE infectivity using these three molecular components.

A concurrent study by Kim and colleagues reported that prions able to induce disease in wild type hamsters can be generated from purified bacterial $rPrP^{C}$ in the absence of any mammalian co-factors using prion-seeded rPMCA [38]. This rPMCA product showed variable attack rates upon inoculation into hamsters and therefore contained low levels of infectivity (incubation time from 119 to 401 days) on the first passage. However, upon second passage all animals became ill with an average incubation period of about 80 days. Lesion profiling indicated that rPMCA had altered the strain characteristics of prions (263K strain) that were initially used to seed the serial rPMCA reactions.

The modulation of conversion of PrP^{C} into PrP^{res} by cofactors was also studied by Abid et al. by using a heterologous PMCA reaction [39]. Their results suggest that the conversion factor involved in prion replication is present in several tissues (e.g., brain, liver, kidney, heart) from different mammalian species and absent in total extracts from other evolutionary lower species such as bacteria and drosophila. This cofactor was found within lipid rafts and most likely was neither a protein nor other molecule that can be denatured by heat. Furthermore, they present evidence that when nucleic acids were depleted from brain homogenate, some other factor promoted the PrP conversion, suggesting that more than one type of molecule can act as a cofactor. In a similar study, using both hamster and mouse PMCA, Deleault and colleagues described species specific difference in the use of cofactors for PrP^{Sc} propagation [40]. They reported that in the case of mouse PMCA only brain and liver homogenates appeared to contain the conversion cofactor, which also appeared to be protease-resistant and heat stable.

To investigate the role of PrP^{C} glycosylation in modulating conversion efficiency Nishina and colleagues tested the ability of un-, mono-, or diglycosylated PrP^{C} to support prion amplification using both hamster and mouse the PMCA reactions [41]. Their data shows that, whereas unglycosylated mouse PrP^{C} is

required to propagate homologous RML prions, diglycosylated PrP^C is necessary to propagate hamster Sc237 prions, suggesting that the stoichiometry of the PrP^C glycoforms influences the efficiency of PrP^{res} formation in vitro. However, more recently, the Supattapone lab also used PMCA to show that PrP glycosylation is not necessary for strain-specific neurotropism [42].

4 Prion Strains and Species Barrier Studies Using PMCA

Prion strains are characterized by distinct incubation periods, clinical symptoms, and brain lesion profiles, as well as differences in biochemical features of PrP^{res} (e.g., electrophoretic mobility, glycoform pattern, infrared spectrum, and conformational stability). Prion strains (or mixtures of strains) can usually be serially passaged stably in a host of a given species and genotype. However, under some circumstances, new strains or mixtures of strains can arise, especially after passage from one host genotype to another. A wealth of evidence suggests that the properties of prion strains are usually maintained by the faithful propagation of different conformers and/or aggregation states of PrP^{Sc} [8, 43–46]. However, the occasional biological instability of prion strains implies that propagation of such conformational states can be subject to permutation, most notably when the prion seed has to act on heterologous PrP^C molecules.

Several PMCA studies support this concept of prion strain propagation. Castilla et al. showed that PMCA generated PrP^{res} seeded with five different murine and four human prion strains retained their specific biochemical properties and, upon injection into wild type animals, the PMCA generated PrP^{res} caused disease with features comparable to the parental strain [47]. Green and colleagues reported that features of the 04–22412 CWD prion strain were kept after PMCA reaction [33]. Collectively, these data are consistent with the idea that prion strain features are encoded, at least to a large extent, by the PrP^{res} conformation. Furthermore, Green and colleagues describe the adaptation of the RML mouse prion strain to Tg (CerPrP) mice, overcoming the mouse–cervid species barrier, and creating a new prion strain using PMCA [33]. In a similar study, Castilla and colleagues describe the generation of new prion strains by hamster–mouse interspecies PMCA amplification [48]. In particular, hamster PrP^C substrate and mouse brain-derived PrP^{Sc}, or vice versa, produced new prion strains which caused diseases with pathological and biochemical features that were unlike those of other known prion strains. Barria and colleagues developed mouse and hamster PMCA reaction conditions that allowed spontaneous generation of PrP^{res} in the absence of initial seeding with PrP^{Sc} [49]. The spontaneous PrP^{res} was infectious in wild type animals but caused a new disease phenotype, suggesting the creation of a novel prion strain. Finally, the 263K scrapie-seeded recombinant PrP prions propagated in rPMCA produced distinct lesion profiles through two passages in vivo, providing evidence that rPMCA with $rPrP^C$ substrate alone with no mammalian cofactors lead to stable changes in strain characteristics [38]. These studies indicate that new prion strains can be generated with interspecies PMCA, unseeded PMCA, or PMCA using solely

$rPrP^C$ as substrate. Usually, when amplifying PrP^{Sc} the PMCA maintains the strain features of the initial seed, probably through precise templating of the PrP^C misfolding process towards the formation of an exact replica of itself. When the PMCA is carried out in the absence of seed, with a heterologous or recombinant PrP^C substrate, additional conformational options presumably become available which enhance the likelihood of forming a new prion conformer or strain.

Collectively, and remarkably, PMCA-based prion propagation mimics prion propagation in vivo to the extent that one can observe not only the stable propagation of prion strains within a given host, but also the permutation of strains and the spontaneous generation of new strains. However, as prion propagation seems largely to be a protein folding problem, we would not expect PMCA reactions to recapitulate all aspects of prion strain propagation and transmission barriers that are seen in vivo. In intact cells or tissues, interactions between PrP^{res} and PrP^C are highly constrained in three dimensions by GPI anchoring to membranes and by localized interactions between the PrP isoforms and other molecules in their physiologically controlled microenvironments. In contrast, PMCA reactions occur in detergent lysates or extracts in which most such constraints on intermolecular interactions are removed.

5 Amyloid Seeding Assay

The Amyloid Seeding Assay (ASA) described by Colby and colleagues is a multiwell plate prion amplification assay that uses thioflavin T (ThT) to detect amplification products [50]. ThT is an amyloid dye that undergoes an enhancement of fluorescence yield when bound to protein amyloid fibrils and is used in this and many other amyloidogenesis assays [51]. The ASA utilizes phosphotungstic acid (PTA) precipitated PrP^{Sc} as a seed and recombinant PrP ($rPrP^C$) stored in 6 M guanidine hydrochloride as a substrate. The final guanidine hydrochloride concentration in the reaction (0.4 M) is such that the substrate is likely in a partially unfolded/destabilized state. Other reaction parameters include incubation at 37 °C, the presence of a 3-mm glass bead in each well to enhance agitation, and continuous shaking of the plate. Notably, the ASA can detect protease-sensitive PrP^{Sc} from transgenic mice over-expressing the PrP (101 L) mutation [28]. ASA applicability to various rodent scrapie experimental models and capability to distinguish between brain samples from sporadic CJD (sCJD) patients and negative control normal brains were described. Furthermore, a 98% correlation of prion detection by ASA and neuropathological lesions in transgenic mice was described. Nevertheless, as noted by the authors, one weakness of the assay is the need to analyze a high number of replicates per sample because of the variability of the kinetics of ThT positive fibril formation. This problem is exacerbated by the fact that, under the ASA conditions, spontaneous (unseeded) $rPrP^C$ fibril formation also occurs, but usually with a longer lag phase than those seen with prion-seeded reactions. As detailed below for the real time QuIC assay, spontaneous fibrillization can be largely avoided under other reaction conditions.

6 Quaking-Induced Conversion Reactions

To avoid technical complexities associated with sonication in PMCA reactions, new assays were developed by Atarashi and colleagues in which sonication was substituted by intermittent shaking as a means to break up prion protein aggregates and produce new PrP seeds in reaction tubes [52–55]. Such shaken conversion reactions have been dubbed Quaking-Induced Conversion (QuIC) reactions. The first-generation QuIC reactions, herein abbreviated Standard QuIC or S-QuIC, were developed as individual microtube-based reactions that contained detergents and used hamster-adapted 263K scrapie as a seed and hamster $rPrP^{C}$ as substrate [53]. As with the rPMCA [26], scrapie seeds induced the conversion of $rPrP^{C}$ to a specific set of proteinase K-resistant bands ($rPrP\text{-}res^{(Sc)}$) that were visualized on immunoblots. The ability to detect as little as 100 ag PrP^{Sc} was demonstrated. Through careful selection of reaction parameters such as shaking regimen, detergent concentrations, incubation time, and reaction temperature, virtual elimination of spontaneous (unseeded) conversion of the substrate to proteinase K-resistant product ($rPrP\text{-}res^{(spon)}$) seed can be achieved (Fig. 2).

S-QuIC has been used successfully to discriminate between scrapie affected and control hamsters using CSF [53] or nasal lavages [56]. The assay was also applied to the detection of prion seeding activity in brain samples from scrapie affected sheep and a human vCJD patient [54]. Furthermore, good discrimination between CSF samples from scrapie positive and normal sheep was observed.

To address limitations of S-QuIC and ASA, Atarashi, Wilham, and colleagues developed a new prion-seeded rPrP conversion assay that combines features of the ASA (i.e., multiwell plate format and ThT detection of conversion products) and the S-QuIC (e.g., intermittent shaking, $rPrP^{C}$ preparation, and lack of chaotropic salts) [52, 55]. This new assay was called Real-Time (RT) QuIC, or herein RT-QuIC, because of its ability to monitor almost continuously the progress of the QuIC reaction in a shaking, temperature-controlled fluorescence plate reader. As with the ASA [50], the multiwell plate format makes the RT-QuIC more amenable to high-throughput testing of samples. However, in contrast to the ASA, the RT-QuIC conditions can, depending on the $rPrP^{C}$ substrate, virtually eliminate the problem of unseeded, prion-independent amyloid formation. The prion-seeded RT-QuIC conversion products were similar to those previously described with S-QuIC [53, 54] and showed distinct PK-resistant bands of ~20, 18, 14, and 13 kDa, while control reactions seeded with normal tissue had virtually no PK-resistant products. Circular dichroism (CD) and Fourier transform infrared (FTIR) studies of the RT-QuIC substrate (hamster $rPrP^{C}$ 90–231) and conversion product indicate that the prion-induced structural changes in $rPrP^{C}$ shared some similarities with those occurring in vivo upon conversion of PrP^{C} to PrP^{Sc}. Thus, RT-QuIC has promise not only as a prion detection assay, but also as a tool to study the mechanism of prion-induced PrP conversion.

Wilham and colleagues also describe the use of RT-QuIC to quantitate prion seeding activity in biological samples. Serial dilutions of a given sample are used as

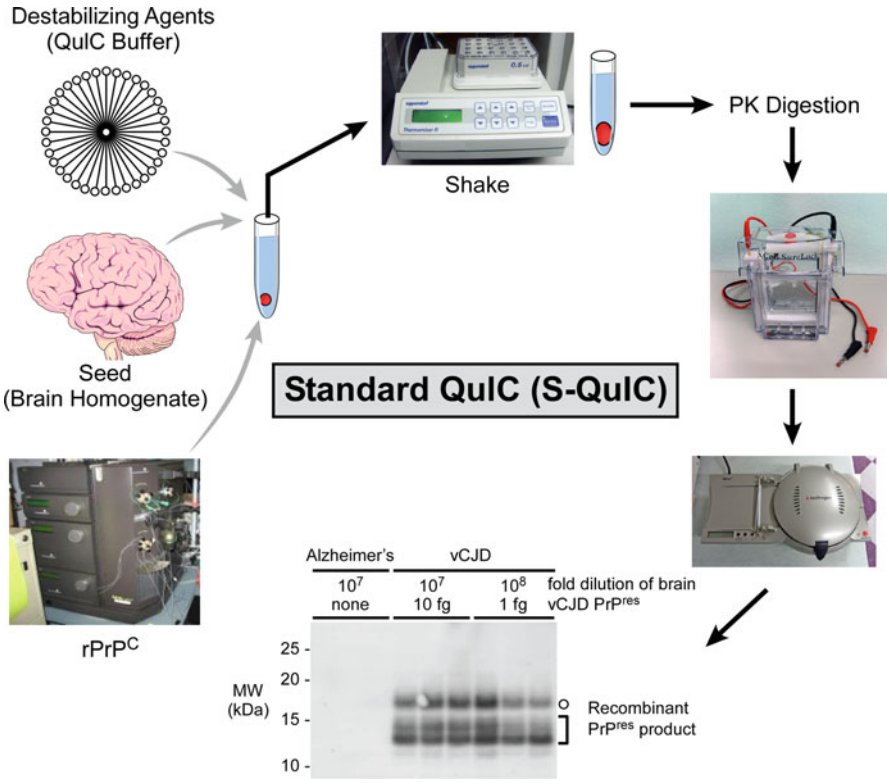

Fig. 2 Standard Quaking Induce Conversion Reaction S-QuIC. The components of the S-QuIC ($rPrP^C$ substrate, brain homogenate seed and conversion buffer) are represented. The final conversion products from reactions seeded with vCJD brain homogenate dilutions containing the indicated amounts of PrP^{res} or Alzheimer's disease (AD) negative controls are shown. Hamster $rPrP^C$ (residues 23–231) was used as substrate and single amplification round reaction proteinase K (PK)-digested products were analyzed using antiserum R20 (C-terminal epitope). *Open circles* mark 17-kDa fragments and *brackets* indicate the lower molecular weight bands (10–13 kDa). Immunoblot adapted from [54]

seeds and the seeding dose (SD) giving 50% ThT-positive replicate reactions (SD_{50}), i.e., the 50% endpoint dilution, is estimated. The SD_{50} is analogous to the 50% lethal dose (LD_{50}) determined in an endpoint dilution animal bioassay. As is commonly done in determining LD_{50} values, the estimation of SD_{50} values can be aided by using Spearman–Kärber [57] or Reed–Muench [58] analyses. This end-point dilution approach to prion quantification is potentially applicable to any prion-seeded amplification assay (e.g., PMCA, rPMCA, ASA). With the RT-QuIC, SD_{50} concentrations obtained for four hamster scrapie brain homogenate stocks were comparable to LD_{50} concentrations obtained with hamster end-point dilution bioassays, indicating similar sensitivities for these two types of assays. However, RT-QuIC has several major advantages over animal bioassays, including practicality, high-throughput potential, rapidity, and reduced cost (Fig. 3).

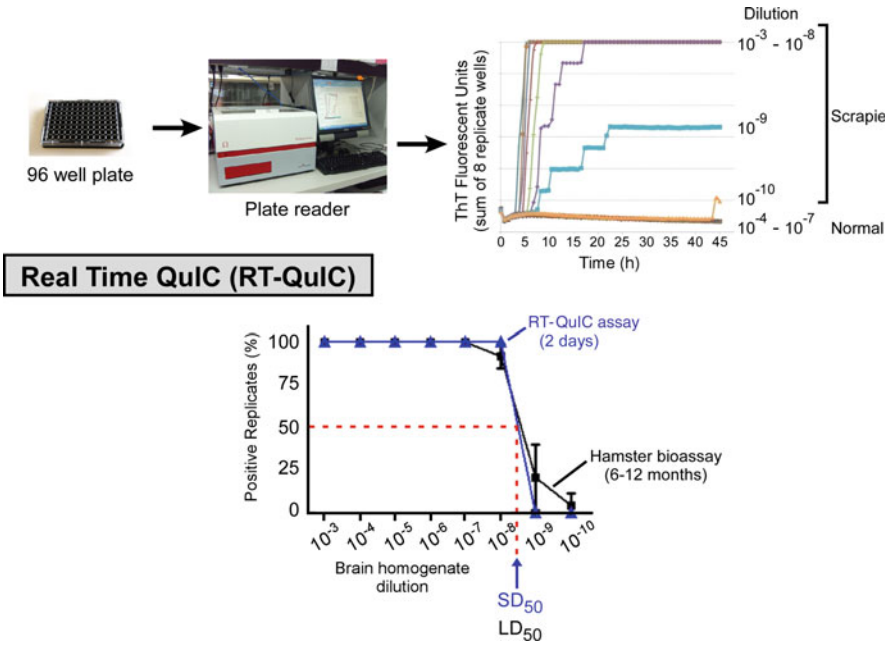

Fig. 3 Diagram of Real Time QuIC RT-QuIC and comparison of end-point dilution titrations of scrapie brain homogenate by RT-QuIC with animal bioassay. (*Top panel*) RT-QuIC analysis of normal and scrapie brain homogenate (BH) dilutions using hamster (90–231) $rPrP^C$ as a substrate. (*Bottom panel*) Comparison of hamster brain homogenate end point dilution titrations by RT-QuIC and animal bioassay. The Spearman–Kärber estimate of the SD_{50} (i.e., seeding dose giving sufficient Thioflavin T fluorescence in half of the replicate wells) per 2 μL of neat brain tissue is indicated. Graphs adapted from [55]

Quantitation of prions in CSF samples from scrapie positive hamsters by RT-QuIC gave SD_{50} values of $10^{5.6}$ and $10^{4.7}$ per mL, respectively. Detection of prions in brain samples from TSE positive sheep and deer was also described. One important version of RT-QuIC has been shown to have 81% sensitivity and 100% specificity in discriminating sporadic-CJD and non-CJD patients based on CSF samples [52].

Of particular interest are prion amplification assays that are capable of detecting prions in blood components such as plasma. However, blood typically has extremely low prion concentrations (i.e., ~13 LD_{50} per mL [59]), and contains inhibitors of some of the most sensitive tests such as PMCA [19] and another assay [60]. Recently, we integrated antibody 15B3-based immunoprecipitation with QuIC reactions to increase sensitivity and isolate prions from inhibitors such as those in plasma samples [61]. Moreover, replacement of the $rPrP^C$ substrate after ~24h in RT-QuIC reactions substantially improved the speed and sensitivity of the assay. Coupling of the immunoprecipitation and substrate replacement steps, which we call enhanced QuIC (eQuIC), dramatically enhanced detection of variant Creutzfeldt-Jakob disease (vCJD) brain tissue diluted into human plasma. 10^{14}-fold dilutions, containing ~2 ag/ml of proteinase K-resistant prion protein, were readily

detected, indicating ~10,000-fold greater sensitivity for vCJD brain than has previously been reported [62]. We also discriminated plasma and serum samples from scrapie-infected and uninfected hamsters, even in early preclinical stages. This eQuIC assay should improve prospects for routine detection of low levels of prions in tissues, fluids or environmental samples.

7 Conclusions

Since the development of the first PrP in vitro conversion reaction [2], much more efficient, continuous, and sensitive prion-seeded conversion assays have been developed. These techniques have been used to investigate prion composition and propagation mechanisms as well as prion strain and transmission barrier phenomena. Moreover, these reactions serve as bases for ultra-sensitive prion detection that should facilitate TSE diagnostic tests and screening assays for medical, agricultural and environmental prion contamination.

A pre-clinical TSE diagnostic test should be sensitive enough to detect minimally infectious or even subinfectious quantities of prions and allow amplification/detection of multiple prion strains in a wide variety of biological tissues. As reported by Wilham and colleagues, inhibitory matrix interference can be overcome by diluting the sample until the reaction is no longer affected by the inhibitors present [55]. Another strategy is to capture and concentrate prions from complex mixtures in a manner that is compatible with amplification/detection as we have recently accomplished using immunoprecipitation [61] and others have reported using steel [62] or magnetic particles [63]. Our studies indicate that, when used in combination with an improved RT-QuIC reaction, immunoprecipitation provides for sensitivities that are several orders of magnitude greater than those obtained using the metallic particles [61].

The fact that so far the infectivity of PrP in in vitro conversion products as been shown to be lower than that of bona fide PrP^{Sc} suggests that we are still missing important information about the conversion process and how to create an infectious prion. The prion-seeded conversion reactions described in this chapter provide valuable tools to investigate these issues.

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Prion Protein and Its Conformational Conversion: A Structural Perspective

Witold K. Surewicz and Marcin I. Apostol

Abstract The key molecular event in the pathogenesis of prion diseases is the conformational conversion of a cellular prion protein, PrP^{C}, into a misfolded form, PrP^{Sc}. In contrast to PrP^{C} that is monomeric and α-helical, PrP^{Sc} is oligomeric in nature and rich in β-sheet structure. According to the "protein-only" model, PrP^{Sc} itself represents the infectious prion agent responsible for transmissibility of prion disorders. While this model is supported by rapidly growing experimental data, detailed mechanistic and structural aspects of prion protein conversion remain enigmatic. In this chapter we describe recent advances in understanding biophysical and biochemical aspects of prion diseases, with a special focus on structural underpinnings of prion protein conversion, the structural basis of prion strains, and generation of prion infectivity *in vitro* from bacterially-expressed recombinant PrP.

Keywords Prion diseases · Prion protein folding · Prion strains · Prion structural biology · Prions

Contents

W.K. Surewicz (✉) and M.I. Apostol
Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, OH 44120, USA
e-mail: wks3@case.edu

Abbreviations

CJD	Creutzfeldt–Jakob disease
GPI	Glycophosphatidylinositol
GSS	Gerstmann–Sträussler–Scheinker disease
HXMS	Hydrogen-deuterium exchange mass spectrometry
PK	Proteinase K
PMCA	Protein misfolding cyclic amplification
PrP	Prion protein
PrP^{C}	The cellular isoform of prion protein
PrP^{Sc}	The pathogenic or scrapie isoform of prion protein
rPrP	Recombinant prion protein
SDSL	Site-directed spin label
TSE	Transmissible spongiform encephalopathy

1 Introduction

A number of neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases and transmissible spongiform encephalopathies (TSEs or prion diseases), are associated with neuronal accumulation of abnormal protein deposits, often composed of amyloid-like fibrils. Collectively, these disorders are often referred to as "diseases of protein misfolding." Despite this general similarity, a unique feature of prion diseases is that, in addition to arising sporadically or by means of inheritance, they are also transmissible. Stanley Prusiner proposed that the infectious pathogen responsible for these diseases is not a virus but a misfolded protein, coining the term "prion" to describe such a highly unusual proteinaceous infectious agent (reviewed in [1]). Within the context of this "protein-only" model, the central molecular event in the pathogenesis of TSE diseases is a conformational conversion of a ubiquitous cellular prion protein, termed PrP^{C}, into a misfolded form, PrP^{Sc}.

Once heretical, the notion that a protein can be infectious is now rapidly gaining acceptance. It is supported not only by a wealth of biochemical data and recent progress in generating TSE infectivity *in vitro*, but also by intriguing findings regarding protein conformation-based inheritance in yeast and other fungi (reviewed in [2]). These advances notwithstanding, a number of questions remain regarding the molecular basis of TSE diseases. Efforts to understand these disorders span many scientific disciplines ranging from neuropathology, animal science, and

cell biology to protein chemistry, molecular biophysics, and structural biology. In this chapter we describe recent advances in the understanding of the biophysical and biochemical aspects of prion diseases, with a special focus on structural aspects of prion protein conversion, the structural basis of prion strains, and efforts to generate prion infectivity *in vitro* from the recombinant prion protein (rPrP).

2 Structural Aspects of the Cellular Form of PrP

The prion protein is ubiquitous in the animal kingdom. It has around 90% sequence similarity across mammals, and although this is lower in other vertebrates, it is still striking that less similar versions of the protein can be found even in reptiles and amphibians [3]. This harkens to the long evolutionary history of PrP^C and the apparently important functional role it must play in the animal kingdom. Despite this inference, however, the function of PrP^C is still poorly understood and, at this time, largely speculative. In this section, we describe the structural properties of PrP^C, and briefly discuss the potential involvement of different structural regions in some of the postulated biological functions and pathogenicity of the protein.

In humans, PrP is encoded as a 253 amino acid long polypeptide. Removal of 22-amino acid N-terminal and 23-amino acid C-terminal signal sequences leaves a functional protein which is 209 amino acids in length and is attached to cell membrane through a glycophosphatidylinositol (GPI) anchor. Other prominent posttranslational modifications include a disulfide bond between Cys179 and Cys214, and N-linked glycosylation at Asn181 and Asn197 (Fig. 1).

Structural and biophysical studies with brain-derived PrP^C have been hindered by relatively low expression levels and the difficulties in obtaining larger quantities

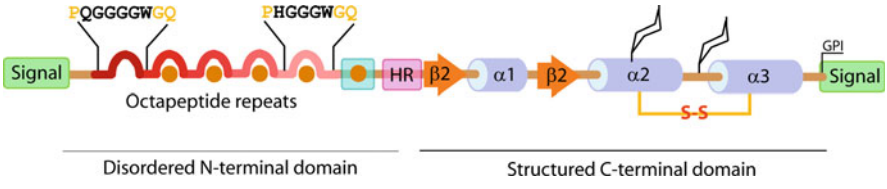

Fig. 1 Schematic representation of human PrP^C structure. Highlighted in *green* are the N-terminal signal sequence and the C-terminal signal sequence (the latter essential for the attachment of the GPI that tethers the protein to the membrane). These signal sequences are absent in the mature form of the protein. The five octapeptide repeats are colored in *shades of red*. The first of these is a "pseudorepeat" lacking a histidine, while the remaining four are a perfect repeats of the sequence PHGGGWGQ known to bind copper ions (represented by *spheres*). A fifth consensus copper binding site is between residues 91 and 111 (highlighted in *cyan*). The "hydrophobic region" containing the palindromic sequence AGAAAAGA is highlighted in *pink*. The consensus secondary structure in the C-terminal domain includes two β-strands (*orange*) and three α-helices (*blue*). The two potential glycosylation sites at Asn181 and Asn197 are shown with attached sugar groups. The disulfide bond between Cys179 and Cys214 is represented by the *yellow* connection between α-helices 2 and 3

of highly purified material. Thus, most structural information about PrP^C has come from studying proteins and polypeptides which have been prepared through recombinant expression in bacteria or chemical synthesis. Despite the lack of posttranslational modifications such as glycosylation and the GPI anchor, rPrP has been shown to be structurally equivalent to PrP^C found *in vivo* [4].

The part of PrP that is structurally best characterized is the C-terminal domain (residues ~121–231), often referred to as the "structured" or "folded" domain. By contrast, the N-terminal part of the molecule is highly flexible, and early nuclear magnetic resonance (NMR) experiments suggested that it is largely disordered [5, 6]. However, recent studies have made substantial advances into the structural understanding of parts of this domain. Below we describe the current state of knowledge about the atomic-level structures of the N- and C-terminal domains.

2.1 The Octapeptide Repeat Region

Much of the N-terminal domain encompasses proline and glycine rich repeats and pseudo-repeats. This region is an evolutionarily conserved motif, though the number of octapeptides can vary between species. In human PrP there are four perfect sequential repeats of the sequence PHGGGWGQ (residues 60–91) and one "pseudorepeat" in which His is substituted with Gln. Under specific conditions (e.g., when bound to certain ligands), these repeats gain a level of order that has allowed for atomic-level insights into their structure.

Within the repeats, histidine and tryptophan residues were found to be essential for the binding of copper ions. The nature and specificity of this binding is illustrated by an X-ray crystal structure of a minimal binding fragment of one repeat (HGGGW), showing the peptide in a β-turn conformation wrapped around and coordinating a Cu^{2+} ion through the main-chain oxygen and nitrogen atoms of glycine residues, and through interactions with histidine and tryptophan side-chains (Fig. 2a) [7]. Spectroscopic data suggest that the same type of structure and coordination of copper as revealed by the crystal structure of the HGGGW segment can be attributed to all four His-containing octapeptide repeats within the full-length PrP [10, 11].

In addition to its ability to bind copper, the octapeptide repeat region has been found to bind polyanions such as glycosaminoglycans, hemin, as well as oligonucleotides and nucleic acids (reviewed in [12, 13]). Recent solution NMR studies have determined structures for the octapeptide repeats in association with the poly-anionic sulfonate glycan (Fig. 2c) [9]. This structure shows that the four histidine containing octapeptide repeats order around the ligand in β-turn conformations, similar to that found in the copper bound state. Insight into the structural properties of a ligand-free octapeptide repeat region were also provided by NMR spectroscopy, suggesting that there is an equilibrium between the β-turn conformation and an unfolded state, and that at neutral pH this equilibrium shifts towards the

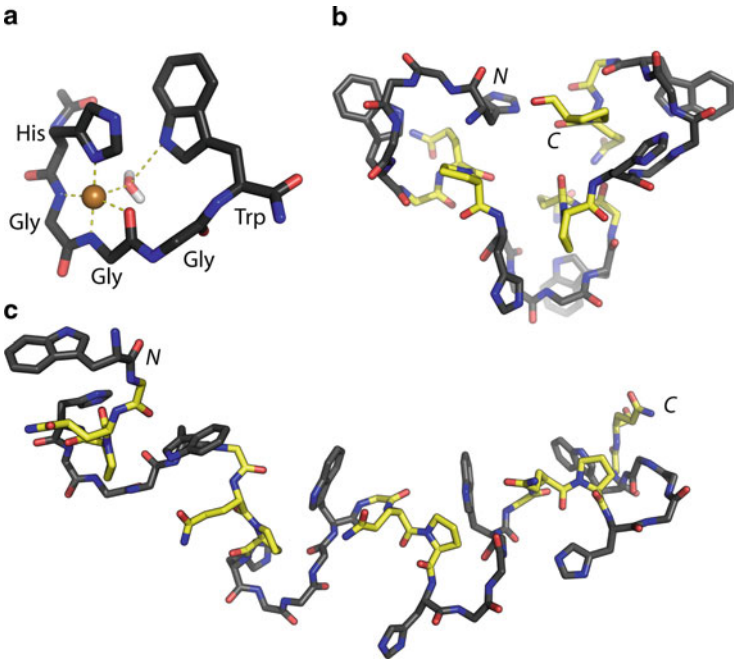

Fig. 2 Atomic structures of octapeptide repeats. (**a**) X-ray crystal structure of copper bound to segment HGGGW [7]. The copper ion is represented as a *sphere* and coordinated through a nitrogen of the histidine side-chain, main-chain nitrogen and oxygen atoms of glycine residues, and a water molecule held in place by hydrogen bonding to the tryptophan side-chain. Coordinates were obtained from "http://www.chemistry.ucsc.edu/~glennm/." (**b**) Solution NMR structure of three octapeptide repeats at pH 6.2 (pdb code 1OEI) [8]. (**c**) Solution NMR structure of four octapeptide repeats bound to a sulfate glycan substrate (pdb code 2KKG) [9]. In all three panels, carbon atoms of the sequence HGGGW are colored in *black* to highlight the conformation of this copper binding motif under different physiological conditions. The carbon atoms of the remainder of the octapeptide repeat sequence GQP are colored in *yellow*. Nitrogen atoms are colored in *blue* and oxygen atoms are colored in *red*

folded state (Fig. 2b) [8]. The implication of all these studies is that, although the repeats have a propensity to be disordered, they can form β-turn conformations under physiologically relevant conditions.

Interestingly, the octapeptide repeat region does not appear to be essential for prion infectivity [14]. Although this region has been hypothesized to play a role in self-association of the prion protein [15, 16], it is not part of a β-sheet rich core of the infectious PrP^{Sc}. Nonetheless, it is perplexing that germline insertion of extra octapeptide repeats result in familial prion diseases in humans [17]. This has been corroborated in mouse models in which the insertion of extra octapeptide repeats causes a spontaneous and progressive neurodegenerative disease [18–20]. Although not essential for pathogenicity, the octapeptide repeat region appears to play a role in modulating the conversion of the protein into the pathogenic form.

2.2 The C-Terminal Domain

More than a dozen structures revealing the atomic details [21–27] of the wild-type and variants of human PrP have been solved by solution NMR and X-ray crystallography. In addition to the human protein, various structures of PrP from at least 14 other vertebrates have been determined utilizing solution NMR [28–30]. These range from disease relevant species, such as cows [31] and sheep [32], to species which have never been found to harbor the disease, such as frogs [33]. Collectively, these studies reveal a consensus structure composed of three α-helices and two short β-strands which form an antiparallel β-sheet. Helices 2 and 3 form the bulk of the structure and are covalently bridged by the characteristic disulfide bond between Cys179 and Cys214 (residue numbering according to human PrP sequence). PrP^C has an unusually small hydrophobic core, which is mainly found between α-helices 2 and 3, and to a lesser extent between helix 1 and small β-sheet which wrap around helix 3 (Fig. 3a). The surface of the protein is decorated by many hydrophilic and charged residues reflective of the sequence of the C-terminal domain.

Although PrP structures from different species are strikingly similar, an intriguing difference in the dynamics of a loop between β-strand 2 and α-helix 2 (residues 165–175 using human PrP numbering) has been observed (Fig. 3d), providing insight into a region of potential importance for pathogenicity and barriers to disease transmission across species. While this loop is highly flexible in most species, it is more conformationally constrained in the PrP structure from cervids such as deer and elk [35]. This rigidity was shown to be fully controlled by two amino acid residues at positions 170 and 174, which in cervids are occupied by Asn and Thr, respectively [35], making this part of the cervid PrP sequence unique among the animal kingdom. Intriguingly, when these cervid specific amino acids were substituted into mouse PrP, the transgenic mice expressing such a hybrid protein developed a spontaneous form of prion disease [36]. Furthermore, it was found that similar loop structures in PrP of the donor and recipient correlate with efficient disease transmission, whereas dissimilar loops correlate with transmissibility barriers [37]. These observations clearly point to the role of loop rigidity in prion disease susceptibility and transmission barriers, though other structural features and specific amino acid residues are obviously also important.

Interestingly, the majority of pathogenic germline mutations that have been linked to cases of inherited human prion diseases appear within the C-terminal domain, most of them in the region encompassing helix 2 and 3 and the loop between them [38]. Notably, many of these mutations neutralize charges (D178N, D202N, E211Q), introduce charged residues (T188K, T188R, H187R, Q217R), or reverse the charge of residues from acid to basic or vice versa (E196K, E200K). Although some pathogenic mutations appear to affect intramolecular salt bridges between side chains or have other minor structural effects (reviewed in [39]), the available solution NMR structures of mutants E200K [21], Q212P [22], and H187R [40], along with crystal structures of mutants D178N and F198S [23], reveal only

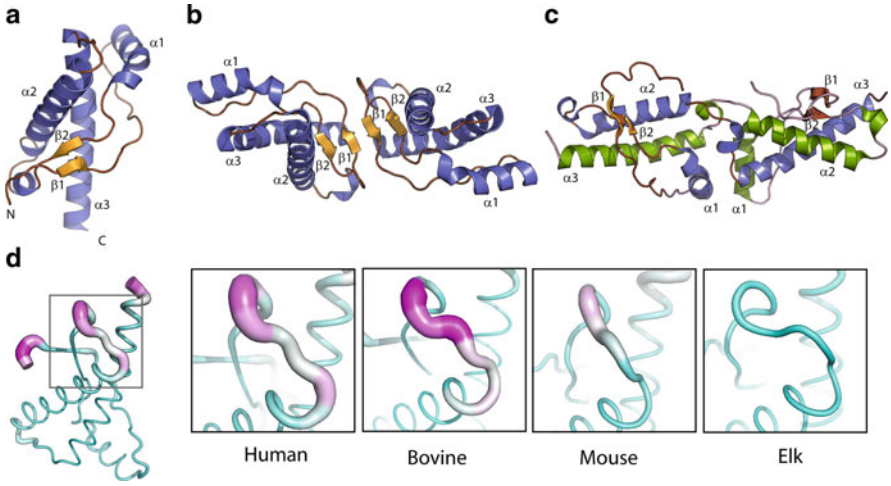

Fig. 3 Atomic structures of the C-terminal domain of PrP^C based on structural data for recombinant PrP. (**a**) The consensus structure of monomeric human PrP^C determined by X-ray crystallography (pdb code 3HAK) [23]. Alpha-helices are colored in *blue* and β-strands in *orange*, with connecting loops in *brown*. Secondary structure elements are labeled (β1–α1–β2–α2–α3, from N- to C- terminus). (**b**) A dimeric interaction between symmetry related molecules within PrP^C crystal structures is mediated by the extension of the native two-stranded β-sheet into an four-stranded antiparallel β-sheet (pdb code 1UW3) [34]. (**c**) A domain- swapped dimer of PrP^C (pdb code 1I4M) [24]. One molecule has the same coloring scheme as in panels (**a**) and (**b**), while in the other molecule α-helices are colored *green* and β-strands *red*. Helix α3 is swapped between the two molecules, forming a disulfide bond with α2 of another molecule. (**d**) On the *left* is a solution NMR structure of human PrP^C (pdb code 1QLZ) [26]; although rotated it is in the identical conformation to the structure in panel (**a**). The "sausage" representation shows the flexibility of the protein as indicated by the thickness of the chain and color. *Cyan* is representative of little backbone dynamics and *magenta* represents higher flexibility. A box over this structure highlights the loop residues 165–175 between β2 and α2. The *insets to the right* show this region in solution structures of human, bovine, mouse, and elk PrP^C (pdb codes 1QLZ, 1DX1 [31], 1XYX [35], and 1XYW [35], respectively). This loop in elk PrP is relatively inflexible when compared to PrP from other species

relatively minor structural differences, providing rather limited insights into the mechanism by which familial mutations facilitate the pathogenic process.

Crystal structures of PrP have been few and far between by comparison to those which have been derived by NMR techniques. To date, crystal structures have been determined only for a few variants of human and sheep PrP [23–25, 34, 41]. Intriguingly, some of these structures reveal two types of homo-dimeric interactions that can exist between PrP molecules, providing a glimpse into the structural repertoire of PrP and the interactions that may be relevant to the conversion into the pathogenic state. One of these interactions is the pairing of the two-stranded antiparallel β-sheet with a symmetry related PrP molecule in the crystal lattice to form an extended four-stranded β-sheet (Fig. 3b). Examples of this interaction have now been seen in multiple structures of human and sheep PrP^C, arguing that it is not an artifact [23, 25, 34]. This extended β-sheet is particularly notable because the

interaction between molecules centers on residue 129, the site of a Met/Val polymorphism in the human population that affects the susceptibility to prion disease [42–44]. Hence, it has been suggested that this interaction may play a role (possibly as a nucleation site) in the $PrP^{C} \rightarrow PrP^{Sc}$ conversion [25, 30, 34].

The second type of interaction found in the majority of human PrP variants crystallized to date is the symmetrical exchange of helices 2 and 3 between a pair of PrP molecules in a phenomenon called "domain swapping" (Fig. 3c) [23, 24]. The formation of such dimers requires the breaking and reforming of two covalent disulfide bonds and implies an intermediate state of the protein under which such an exchange could happen. Domain swapping has also been proposed as a mechanism of ordered protein aggregation for some amyloidogenic proteins [45–48]. However, at present it is not clear whether any domain swapped form of PrP exists *in vivo*.

3 Copper Binding

As mentioned in Sect. 2.1 describing the octapeptide region, prion protein binds Cu^{2+} ions, suggesting that it may play a role in copper homeostasis. A number of studies have explored functional aspects of this interaction, postulating that PrP^{C} acts as a transporter of copper, a sink for excess copper, a copper-dependent receptor, or a scavenger of Cu^{2+} generated free radicals [49, 50]. However, despite these leads, there is currently no consensus regarding the specific physiological function of copper binding. Another hotly contested issue is the affinity of this interaction, with reported dissociation constants for Cu^{2+} binding to PrP^{C} ranging from micromolar to femtomolar values [51–55].

A structural understanding of the coordination of copper to the octapeptide repeats was discussed above. However, octapeptide repeats do not account for all of the copper binding functionality of PrP. A second region implicated in Cu^{2+} binding includes two histidine residues, His96 and His111 [51, 56, 57]. Despite a lack of the same consensus sequence as the octapeptide repeats or strict structural evidence for binding, this site has been reported to have nanomolar affinity for copper, possibly higher than the octapeptide repeats [58]. Furthermore, yet another Cu^{2+} binding site has been suggested within the C-terminal domain between residues 121 and 231 [59], although its precise location is unclear (reviewed in [60]).

Although copper binding seems to be involved in the normal physiological function of PrP^{C}, the interplay between copper binding and disease pathogenesis is not clear. The binding sites C-terminal to the octapeptide repeats are potentially significant as they are within the 90–230 region, a part of the protein critical for prion infectivity. This raises the possibility that the binding of copper could modulate the conversion of PrP^{C} to PrP^{Sc}. However, animals that were dosed with excess copper [61] and those that were treated with metal chelators [62] both had a delayed onset of disease symptoms, pointing to a complex relationship between copper and the pathogenic process in TSE diseases.

4 Folding of the Prion Protein Monomer and Partially Structured Intermediates

A number of recent studies focused on understanding the folding pathway and energy landscape of the monomeric form of the prion protein, with the expectation that such studies could provide insight into initial events in the conversion process. A question of particular interest relates to the involvement of specific intermediates in the folding of PrP. While partially structured monomeric intermediates are believed to play a central role in fibrillation of many amyloidogenic proteins [63], detection and characterization of such intermediates for PrP proved to be far less than straightforward, though significant progress has been made in recent years.

One approach to studying the mechanism of protein folding involves kinetic stopped-flow experiments in which the progress of the folding or unfolding reactions is followed by optical probes after rapid dilution of the protein into a buffer containing different concentrations of chemical denaturants. Such studies with PrP proved to be challenging since the kinetics of prion protein folding is extremely fast [64]. This could be explained by the presence of native-like residual structure in PrP under denaturing conditions as observed by NMR spectroscopy [65]; this residual structure could act as a template accelerating the folding process. While initial stopped-flow experiments with mouse rPrP failed to detect any folding intermediates [64], more recent studies using human rPrP revealed that, during refolding from urea, the protein populates a partially structured intermediate which precedes the rate-limiting formation of the native state [66, 67]. The accumulation of such a partially structured intermediate was confirmed in subsequent ultrafast continuous-flow experiments [68]. Detailed analysis of kinetic data revealed that, while at neutral pH this intermediate is of relatively low stability, it becomes significantly stabilized (and, thus, more populated) under mildly acidic conditions [67, 68]. This stabilization is of potential relevance to prion disease pathogenesis as some reports suggest the involvement of acidic compartments in the $PrP^{C} \rightarrow PrP^{Sc}$ conversion *in vivo* [69].

Partially structured folding intermediates are typically characterized by an increased exposure of the polypeptide backbone to solvent and relatively high hydrophobicity. This, combined with much higher stability of the intermediate as compared with the fully unfolded prion protein, renders this intermediate state a likely candidate for a monomeric species that is directly recruited into the aggregates state and, eventually, converts to the PrP^{Sc} structure [67, 68]. Such a scenario was further explored in studies with human PrP variants carrying point mutations associated with inherited prion diseases [66]. Because these amino acid substitutions produce a lethal dominant condition, understanding how they affect the folding pathway of PrP could provide important clues regarding the mechanism of the $PrP^{C} \rightarrow PrP^{Sc}$ conversion process. Interestingly, approximately two-thirds of familial mutations map to the helix 2/helix 3 region, and the folding pathway of PrP was found to be very sensitive to even minor modifications of the contact surface between these two α-helices [70]. Kinetic studies of Apetri et al. [66] found that, in

a vast majority of cases tested, familial mutations result in a pronounced stabilization (and thus increased population) of the folding intermediate, regardless of the effect of these mutations on the global thermodynamic stability of PrP [71, 72]. Furthermore, in each case, the population of the intermediate is at least one order of magnitude higher than that of the fully unfolded state. Based on these observations it was proposed that many familial prion diseases may arise from an increased population of a partially folded intermediate, by a mechanism in which the population of such an intermediate becomes sufficient to initiate the aggregation process in the absence of exogenous seeds, leading to de novo formation of PrP^{Sc} [66].

Monomeric folding intermediates of the prion protein were also probed under equilibrium conditions. Early studies described an acid-induced β-sheet rich form of rPrP that was suggested to represent a monomeric equilibrium intermediate [73, 74]. However, it was subsequently shown that the β-sheet-rich species observed in these experiments is not monomeric but, in fact, represents an aggregated form of rPrP [75, 76]. More conclusive hints regarding the presence of monomeric intermediates under equilibrium conditions were provided by NMR spectroscopy, suggesting that, in addition to the native and fully unfolded random coil states, at least three different partially folded forms of PrP may exist: a perturbed native state characterized by conformational rearrangement of the C-termini of helices 2 and 3, a partially denatured state characterized by a hyperstable core consisting of at least ten residues centered on the disulfide bond bridging helices 2 and 3, and an even less folded species with characteristics similar to that of a molten globule state (Fig. 4) [65]. Furthermore, NMR measurement of bovine PrP unfolding in urea revealed markedly different stabilities for different residues monitored, implying that the denaturation process involves many microscopic intermediates [77]. It was also reported that those equilibrium unfolding curves of hamster PrP monitored by far-UV circular dichroism are dramatically different from those monitored by fluorescence of engineered Trp residues, suggesting the presence of a highly populated folding intermediate [78]. However, the latter observation is at odds with previous reports from other laboratories, which found a perfect coincidence of PrP unfolding curves as monitored by circular dichroism and Trp fluorescence [64, 66].

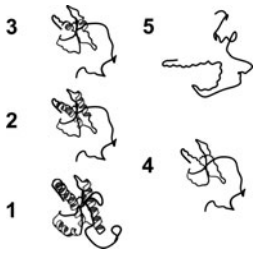

Fig. 4 Proposed intermediate states during the unfolding of PrP90–231. Schematic showing the intermediate states of PrP as measured by NMR, starting from natively folded monomer (1) and progressing into the unfolded state (5). Reproduced from [65] with permission from the author and the journal *Biochemistry*

Further insight into the folding pathway of PrP was provided by high pressure spectroscopy [79–82], a method particularly well suited for detection of rare conformers that might exist under physiological conditions. These studies identified a non-native metastable intermediate characterized by preferentially disordered α-helices 2 and 3 [79], a region of PrP found to undergo slow fluctuations [83]. This intermediate appears to be populated at approximately 1% at ambient pressure and temperature, and it was suggested that it could represent a monomeric precursor on the pathway of the $PrP^{C} \rightarrow PrP^{Sc}$ conversion [79]. Another study identified a second potential intermediate, which is characterized by melting of the structure within the loop between β-strand 1 and α-helix 1 [82].

5 Prion Protein Conversion and the Biophysical Properties of PrP^{Sc}

5.1 Models of Prion Replication

Within the context of the protein-only model, the central molecular event in the replication of mammalian prions is the self-propagating conformational conversion of PrP^{C} to the misfolded PrP^{Sc} form. Two conceptually different mechanisms have been proposed for this protein-based replication process. According to the heterodimer refolding model (also known as the template assistance model), PrP^{Sc} exists as a monomer that is thermodynamically more stable than PrP^{C}, but this thermodynamically favored conformer is kinetically inaccessible [84]. In this scenario, a critical step in the conversion is the formation of a heterodimer between PrP^{Sc} and PrP^{C} (or a partially destabilized folding intermediate of PrP^{C}), with PrP^{Sc} acting as a monomeric template that catalyzes the refolding of PrP^{C} to a thermodynamically more stable PrP^{Sc} conformation (Fig. 5a). While such a mechanism is plausible, there is no experimental evidence for the existence of a stable PrP^{Sc} monomer. By contrast, available data indicate that prion protein conversion is intimately associated with the aggregation process [75, 76, 85–87], and the infectious entity is oligomeric in nature [88].

A model consistent with the oligomeric nature of PrP^{Sc} is the so-called nucleated polymerization mechanism [89], according to which the conversion between PrP^{C} and PrP^{Sc} is reversible, but the PrP^{Sc} monomer is much less stable than PrP^{C} (i.e., the equilibrium is strongly displaced toward PrP^{C}). Stabilization of PrP^{Sc} occurs only upon formation of a stable oligomeric nucleus, formation of which is thermodynamically not favorable. However, once the nucleus has formed, monomeric PrP^{C} could efficiently add to it, adopting the conformation of PrP^{Sc} (Fig. 5b). The rate-limiting step in this mechanism is, thus, not conformational conversion itself but the nucleation step. This step, responsible for the lag phase in the spontaneous conversion, can be bypassed by addition of preformed PrP^{Sc} seeds. For stable propagation of the prion state *in vivo*, the rate of this template-mediated aggregate

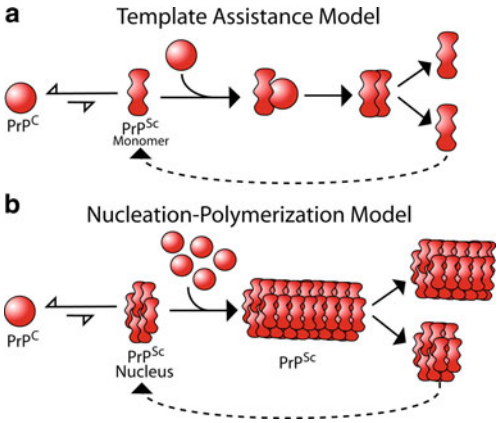

Fig. 5 Models of prion replication. (**a**) The template assistance model predicts that a PrP^{Sc} monomer is more stable than PrP^{C}, but is kinetically inaccessible. In the rare event that a PrP^{Sc} monomer is created spontaneously (or provided exogenously), it can template the misfolding of another PrP^{C} molecule by direct interaction. The *dashed line* shows that the newly created PrP^{Sc} monomer can act as another seed to formation of PrP^{Sc}. (**b**) The nucleation polymerization model predicts that barrier to prion protein conversion is the formation of a nucleus in which the protein adopts a PrP^{Sc}-like structure. The formation of such a low order aggregate is not favored; however, once it has formed, polymerization from a pool of PrP^{C} molecules can take place efficiently. Fragmentation of the polymer increases the number of ends for the recruitment of PrP^{C} monomers

growth must exceed the rate of biological clearance. A mechanism that could facilitate this process during infection is continuous fragmentation of growing PrP^{Sc} aggregates into smaller particles, providing new "ends" for conformational conversion of additional PrP^{C} molecules. Such fragmentation was found to be important for amyloid formation by many proteins *in vitro*, including the yeast prion protein Sup35 [90–92]. In the case of yeast prions, fibril fragmentation and maintenance of the prion state *in vivo* is believed to be mediated by the molecular chaperone Hsp104 [93]. While there are no known mammalian equivalents of the disaggregating chaperone Hsp104, recent modeling of kinetic data indicates that fragmentation of large PrP^{Sc} aggregates is an essential factor in the replication of mammalian prions as well [94].

5.2 *Biochemical and Biophysical Characteristics of PrP^{Sc}*

Despite decades of research, structural properties of PrP^{Sc} remain poorly characterized. This is, in large part, due to difficulties in the large scale purification of brain-derived PrP^{Sc} and potential heterogeneity of this material. Apart from its oligomeric nature, a defining feature of PrP^{Sc} is an unusually high resistance to degradation by proteolytic enzymes such as proteinase K (PK). The PK-resistant core of PrP^{Sc} is typically associated with the entire C-terminal region starting

at residue ~80–90, often referred to as PrP27–30 [14, 95]. However, in some cases shorter PK-resistant fragments have also been identified. For example, at least four distinct PK-resistant fragments (all of them C-terminal) are distinguishable by Western blot analysis in cases of sporadic CJD [96]. Yet different PK-resistant fragments, some of which are within the middle part of the PrP sequence, are associated with inherited human prion diseases such as GSS [97]. The picture is further complicated by reports pointing to the presence in TSE-infected brains of apparently PK-sensitive abnormal forms of PrP [98–100], though it is not known whether the latter species carry any infectivity.

The PK-resistance of PrP^{Sc} is an easily measurable operational parameter that has played a fundamentally important role in the evolution of concepts regarding the nature of mammalian prions. However, from the perspective of structural biology, this parameter is merely a surrogate of structural information, as PK-resistance may be associated with different structural motifs, and may also result from the presence of additional molecules that associate with PrP^{Sc} and prevent access of the protease to potential cleavage sites.

Low-resolution optical spectroscopic measurements established that the $PrP^{C} \rightarrow PrP^{Sc}$ conversion is accompanied by a major decrease in α-helical content and an increase in β-sheet structure [101–104]. However, the secondary structure of PrP^{Sc} remains controversial, with the estimated content of α-helical structure ranging from 0%, as assessed by circular dichroism spectroscopy [104], to ~0–21%, as inferred from Fourier-transform infrared measurements [101–103, 105]. These low resolution spectroscopic measurements are subject to considerable uncertainty; thus, caution should be exercised when using global secondary structure estimates for constructing specific high-resolution structural models of PrP^{Sc}.

Of particular interest in prion biology are ordered protein aggregates known as amyloid fibrils. These aggregates, which typically display affinity for certain aromatic dyes such as Congo red and thioflavin T, are characterized by a common cross-β core, where β-strands are perpendicular to the long fibril axis and stabilizing hydrogen bonds are parallel to this axis [106]. While in the case of yeast prions it is well established that the infectious entity is represented by classical amyloid fibrils formed by specific yeast proteins [2, 107, 108], the picture for mammalian prions is less clear. Amyloid deposits are often present in humans afflicted with inherited prion diseases such as GSS, and these plaques are typically composed of relatively short internal fragments of PrP that lack the GPI anchor [97, 109, 110]. Furthermore, large quantities of classical amyloid deposits accumulate around blood vessels in the brains of scrapie infected transgenic mice that express a PrP^{C} variant lacking the GPI anchor [111, 112]. When isolated and characterized by electron and atomic force microscopies, these "anchorless" prions were found to be composed of protofibrils approximately 3–3.5 nm in width and with somewhat variable morphologies [113]. By contrast, PrP^{Sc}-containing plaques in many TSE diseases in hosts expressing wild-type PrP^{C} appear to be membrane-associated and lack fibrillar morphology typical for amyloids (reviewed in [114]). It is not clear whether this is due to fundamentally different structural properties of these deposits or merely reflects technical aspects in the imaging of membrane-bound material,

especially since amyloid-like fibrils (prion rods) have been reported for partially purified PrP^{Sc} upon its separation from membranous structures with detergents [115, 116].

While TSE infectivity is typically associated with relatively large PrP^{Sc} aggregates, it is not clear what constitutes the basic infectious unit. A number of reports indicate that prion infectivity (or part thereof) may be preserved upon partial disaggregation by chemical and/or physical treatment of large insoluble PrP^{Sc} polymers into smaller particles [117]. Furthermore, the recent fractionation analysis of detergent-extracted and partially disaggregated PrP^{Sc} shows that that the highest specific infectivity is associated with relatively small oligomeric species, with masses equivalent to 14–28 PrP monomers [88]. It is not clear, however, whether such small infectious particles exist *in vivo*, and whether higher infectivity per mass unit of these particles as compared with larger fibrils is due to fundamental structural differences at the molecular level or merely reflects the larger number of ends available for the recruitment of PrP^{C} monomers.

5.3 *Structural Models of PrP^{Sc}*

One of the most difficult challenges in the TSE field is the determination of the atomic structure of PrP^{Sc}. While a number of approaches have recently emerged to probe the structure of ordered protein aggregates, most of them require the introduction of specific spectroscopic probes; therefore, they are not applicable to proteins derived from mammalian tissue. In the absence of direct experimental data, the structures proposed for PrP^{Sc} are largely based on modeling efforts.

The two frequently discussed models for the structure of the PK-resistant core of PrP^{Sc} are known as the "β-helical" and "spiral" models. The β-helical model was designed to fit electron density from electron micrographs of two-dimensional crystals which were found to form after chemical disaggregation of PK digested mouse prion rods [118, 119]. The model presumes that the N-terminus of the PK-resistant part of PrP^{Sc} (residues 89–175) is in a β-sheet rich conformation [119]. The latter has been modeled by threading these residues onto the scaffold of the β-helical part of a *Streptococcal* urydiltransferase structure [120]. Both native β-strands and α-helix 1 of PrP^{C}, as well as the innately disordered residues which precede them, fit into the rungs of the left-handed β-helix, whereas the two C-terminal α-helices 2 and 3 are assumed to be largely preserved as in the native PrP^{C} structure. The modeled β-helical domains of PrP associate into a trimer, where the native-like α-helices decorate the outside of the assembly providing space for sugar groups at the two glycosylation sites. To explain assembly into amyloid fibrils, the trimeric β-helical motifs are hypothesized to stack on each other, forming an extended β-sheet core along the fibril axis (Fig. 6a). As such, the model is compatible with the cross-β diffraction reported for brain derived prion rods [124]. Recently β-helical structures have been found to constitute the amyloid core of the fungal prion HET-s [125] and have been proposed for the bacterial curli

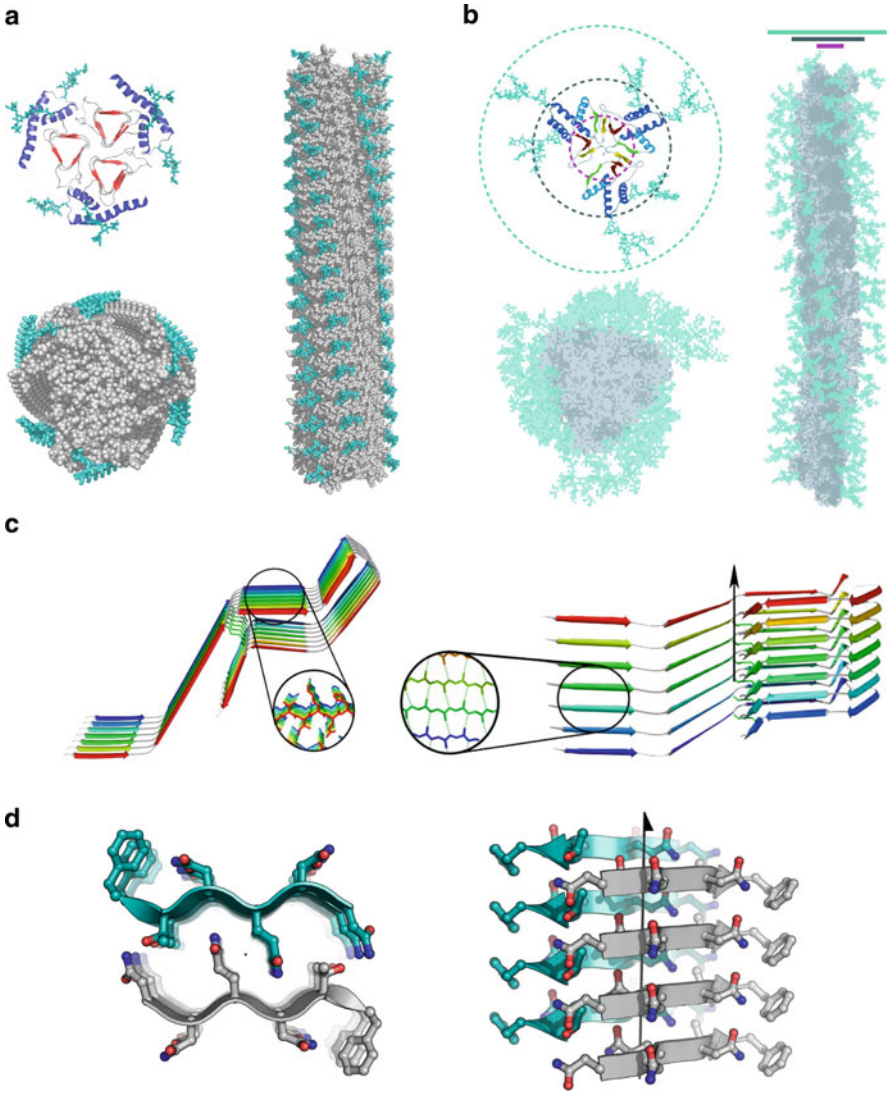

Fig. 6 Structural models of PrP^{Sc} and prion protein amyloid. (**a**) The β-helical model of PrP^{Sc}. *Left top panel*: The region comprising residues 89–175 is wound into the β-helical fold with individual β-strands labeled in *red*, and these β-helices self-associate into the trimeric assembly. α-Helix 3 and major part of α-helix 2 remain in a native-like conformation (*blue*). Oligosaccharides decorate the outside of the trimer (*cyan*). *Bottom left panel*: Space-filling model of the trimer where the protein is shown in *gray* and oligosaccharides in *cyan*. *Right panel*: The trimers stacked into an assembly reminiscent of a protofibril or fibril. Adapted from [119] using the coordinates kindly provided by H. Wille. (**b**) The β-spiral model of PrP^{Sc} protofibrils illustrated using the same panel scheme and color code as in (**a**). Reproduced from [121] with permission of the authors and *Proceeding of the National Academy of Sciences*. (**c**) A parallel, in-register β-structure model for the core of recombinant PrP90–231 amyloid fibrils formed *in vitro*.

proteins [126]. However, experimental data are still lacking to demonstrate that such a structure is indeed present in PrP^{Sc}.

The β-spiral model was derived from molecular dynamics simulations using the monomeric structure of PrP^{C} as a starting point [121]. Reflective of this, the model retains most of the distinguishing features of the native PrP^{C} structure including all three α-helices. However, the results of the simulation suggest that during prion conversion, the two native β-strands elongate into a longer β sheet, while two new β-strands are formed from a loop between strand 1 and helix 1 in the native structure in addition to the disordered residues N-terminal to strand 1. The model hypothesizes that molecules of PrP can interact along these new beta-strands forming intermolecular β-sheets that account for a mechanism of polymerization. To fit into the electron density prescribed by the two-dimensional crystals of disaggregated PrP^{Sc} [118], the basic subunit of the oligomer was also modeled as a trimer. In the stacked assembly of the trimer into a PrP protofibril, the new β-sheets are at a 45° pitch relative to the fibril axis and "spiral" around this axis, giving the model its characteristic name (Fig. 6b). Unlike in the β-helical model, β-sheets are neither continuous along the fibril axis nor exclusively parallel, and therefore by themselves could not account for the cross-β diffraction pattern found for PrP^{Sc}, implying that, at best, this is a model of an intermediate state in which further conformational changes would need to take place during the transformation into PrP^{Sc}-like amyloid.

On the experimental front, the structure of PrP^{Sc} derived from brain tissue of scrapie-infected transgenic mice expressing GPI-free prion protein was recently examined by the hydrogen/deuterium exchange method [105]. In contrast to the models described above, these data indicate that the $PrP^{C} \rightarrow PrP^{Sc}$ conversion involves major refolding of the entire region C-terminal to residue ~80–90, and that this region in PrP^{Sc} consists of a network of β-strands and relatively short turns, with no native α-helices present.

5.4 Structural Studies with rPrP Amyloid Fibrils

There has been great interest in recent years in understanding the biophysical and structural aspects of prion protein conversion using bacterially-expressed rPrP. Depending on experimental conditions, rPrP has been shown to form a variety of oligomeric structures [75, 76, 127–131]. However, of particular interest in the

Fig. 6 (Continued) The *left* part of the panel is a cross-sectional view showing tight interdigitation of side chains, whereas the *right* part of the panel looks along the fibril axis and shows the network of intermolecular hydrogen bonding. Adapted from [122]. (**d**) Atomic structure of segment 173–178 from elk PrP showing a steric zipper motif (pdb code 3FVA) [123]. The two β-sheets that make up the steric zipper interface are colored in *cyan* and *gray*. The *left panel* is a cross-sectional view of the zipper showing the tight interdigitation of side-chains between the sheets into what is known as a dry interface. The panel shows the zipper along the fibril axis

context prion diseases are rPrP amyloid fibrils [132–137], especially since the latter structures are self-propagating in nature and have been shown to cause transmissible neurological disorders in experimental animals (see Sect. 7). Compared to brain-derived PrP^{Sc}, amyloid fibrils formed spontaneously *in vitro* from rPrP23–231 or rPrP90–231 display a significantly shorter PK-resistant core, with N-termini of major PK-resistant fragments mapping to residues 162 and 152/153 [138]. While this PK-resistant region can be extended to encompass the entire ~90–231 segment upon fibril "annealing" by heating in the presence of detergents [139] or brain homogenate [140], or by preparing fibrillar aggregates by a PrP^{Sc}-seeded protein misfolding cyclic amplification (PMCA) procedure [141, 142], it is not clear whether this PrP^{Sc}-like PK resistance results from an ordered conformation in the entire ~90–231 region of rPrP, or rather from interactions with additional molecules that may occlude potential cleavage sites [142].

The structural properties of rPrP amyloid fibrils formed spontaneously by rPrP90–231 were recently examined by a number of biophysical methods including hydrogen/deuterium exchange coupled with mass spectrometry (HXMS) [143] and site-directed spin labeling (SDSL) [122]. These experiments consistently indicate that the systematically H-bonded β-sheet core region maps to residues ~160/170–220, implying that the conversion of PrP to amyloid fibrils must involve a major refolding of the C-terminal domain from α-helix to β-structure. Solution NMR data suggest that some degree of immobilization may also exist in a more N-terminal part (up to residue 145) [144], though this does not necessarily imply the presence of ordered secondary structure in this region. Distance measurements by SDSL revealed that residues within the ~160–220 core region form single-molecule layers that stack on top of one another with a parallel and in-register alignment of β-strands (Fig. 6c) [122]. This type of structural arrangement was subsequently confirmed by solid-state NMR experiments [145]. The parallel, in-register β-structure, a motif fundamentally different from both the β-helical and spiral models discussed above, has been found experimentally for rPrP amyloid fibrils formed both in the presence of denaturants [122, 145] as well as under native-like conditions [137], suggesting that such a structure likely represents a distinct global minimum in free energy landscape. In fibrillar aggregates formed in PrP^{Sc}-seeded PMCA reaction, the β-core region was found to extend at least up to residue 145, with some degree of structural order also present within residues ~117–133 [142].

Interestingly, the structural properties of amyloid fibrils formed by fragments of PrP other than 23–231 and 90–231 appear to be context dependent. In these, different residues were found to define the core region depending on the particular fragment. For example, solid-state NMR experiments indicate that the ordered β-core in fibrils formed by a C-terminally truncated Y145Stop variant of human PrP (huPrP23–144) maps to residue 112–141 [146], i.e., the part of the protein that remains largely disordered in fibrils formed by PrP23–231 or PrP90–231. Furthermore, two solid-state NMR studies have solved structures of PrP fibrils from fragments 106–126 [147] and 109–122 [148], finding them in parallel and antiparallel conformations, respectively. Eisenberg and coworkers have found that small segments from amyloidogenic proteins, including PrP, can form a variety of

parallel and antiparallel structures that can define the general amyloid "fold" [149]. These have been called steric zippers and are characterized by segments being arranged in β-sheets which interact along a self-complementary interface devoid of water (Fig. 6d) [150]. The self-complementary nature of the steric zipper interface has been shown to be prone to distinct structural changes due to subtle changes in amino acids, and has been suggested as one possible explanation for the effects of the prominent human 129Met/Val polymorphism in determining disease susceptibility [151].

6 The Structural Basis of Prion Strains

One of the most interesting, though poorly understood, features of TSE diseases is the existence of multiple prion strains. Different prion strains typically lead to distinct disease phenotypes that are distinguishable by incubation times, clinical symptoms, and patterns of neuropathology [1, 152, 153]. Since multiple strains can be serially propagated within the same species (i.e., hosts with identical PrP gene sequence), the phenomenon of prion strains cannot be attributed to differences in amino acid sequence. Transfer of a prion strain from species A to species B typically results in low attack rates and very long incubation times. However, these incubation times are often greatly reduced, and the full attack rate is restored, in subsequent passages within species B, a phenomenon known as "strain adaptation" (reviewed in [152]). Under some circumstances, prion strains can also "mutate" (i.e., change their phenotypic properties) or undergo selective amplification due to altered environmental conditions [152, 154].

While for conventional pathogens such as viruses the phenomenon of strains can be readily explained by differences in their nucleic acid genome, findings regarding the existence of multiple strains of the prion agent have for many years presented a major challenge to the protein-only model of TSE diseases. The only way to rationalize these findings within the framework of this model is to assume that each strain represents a distinct conformation of PrP^{Sc}. The notion that a protein can exist in many different stable conformations that define transmissible phenotypes was slow to be accepted, especially since PrP^{Sc} was originally thought to represent an abnormally folded monomeric species [84]. Once heretical, this idea became conceptually more acceptable with growing evidence that PrP^{Sc} is polymeric in nature, as such polymeric organization could provide a basis for structural polymorphism.

Initial evidence that strain diversity may be enciphered in the structure of PrP^{Sc} was provided in studies with two strains of transmissible mink encephalopathy passaged in hamsters, designated hyper (HY) and drowsy (DY) [155]. It was found that the PK-resistant core of PrP^{Sc} from DY is ~2 kDa smaller than that from HY, implying that the two PrP^{Sc} strains have different cleavage sites and, thus, different conformations [156]. Following this historically important observation, distinct conformational properties of HY and DY PrP^{Sc} were corroborated in studies

showing strain-specific differences in the exposure of certain epitopes [98], positions and intensities of bands in infrared spectra [157], and conformational stability as assessed by resistance to denaturation by chaotropic agents such as guanidine HCl [158]. Similar differences between PrP^{Sc} types have also been identified for other strains of TSE diseases [98, 159], providing strong support for the conformational model.

This progress notwithstanding, our understanding of the structural basis of mammalian prion strains remains largely at the conceptual level, as properties such as PK resistance are little more than biochemical surrogates of structural information. Distinct positions of infrared bands observed for PrP^{Sc} from HY and DY suggest differences at the level of β-sheet structure. However, the exact nature of these differences and the specific protein regions involved remain unknown. Conformational stability studies, which typically measure the loss of PK-resistance upon exposure to different concentrations of guanidine HCl, demonstrate remarkably large differences between PrP^{Sc} corresponding to different strains [158]. However, the interpretation of these data in specific thermodynamic and/or structural terms is also unclear as it is not known whether denaturant-induced loss of PK-resistance reflects dissociation of PrP^{Sc} into underlying monomers or, rather, is due to conformational changes within the polymer structure. Thus, more discriminating methods and higher resolution approaches are needed to reveal the precise structural features of PrP^{Sc} types that define their strain properties. As a step in this direction, a recent study examined strain-specific structural differences in brain-derived PrP^{Sc} using the method of hydrogen/deuterium exchange, finding that strain properties may be discriminated both by the differences in the overall length of the ordered β-core region in PrP^{Sc} as well as by relatively minor differences in the identity of internal β-strands [105]. Further higher-resolution studies may also help to explain intriguing findings that the pattern of glycosylation (i.e., the ratios of di-, mono-, and unglycosylated forms of PrP) is one of the determinants of prion strains, being faithfully maintained upon transmission. This could be accommodated within the framework of the conformational model by assuming that specific glycosylation patterns modulate the structure or stability of PrP^{Sc}. However, without structural data, such a scenario remains just a hypothesis.

6.1 Lessons from Studying Conformational Polymorphism in Amyloid Fibrils

The notion that prion strains are encoded by different conformations of the same protein is supported by a wealth of data showing structural polymorphisms (or "strains") in amyloid fibrils formed by a number of unrelated proteins such as insulin [160], Alzheimer's Aβ peptide [161], and Sup35 [107, 108]. Importantly, the conformational traits defining individual polymorphic forms are often "heritable" upon seeding, with "daughter" fibrils adopting the conformation of "parent" seeds, even if the reaction is performed under conditions that would normally favor

a different type of conformation in the absence of seeds [160, 161]. For yeast prion protein Sup35, two types of infectious fibrils associated with distinct phenotypes of yeast prion state were found to differ with respect to the length of the ordered β-sheet core [162, 163]. In the case of Aβ fibrils, the differences between polymorphic forms appear to be much more subtle, limited to external quaternary contacts and overall symmetry [161, 164]. Perhaps not surprisingly, polymorphic variability was also observed for fibrils formed by the rPrP. It was observed that fibrils formed under slow rotation, denoted R-fibrils, have a different morphology than those formed under vigorous shaking, denoted S-fibrils, and these distinct morphologies were inherited in seeding experiments under alternative shaking modes [165]. Apart from showing distinct morphologies, the R- and S-fibrils were found to differ with respect to X-ray diffraction patterns and other biophysical properties, suggesting two different architectures of cross-β structure [166].

Conformational polymorphism was also observed for amyloid fibrils formed by the recombinant protein corresponding to PrP fragment 23–144, and studies with this system revealed an intricate relationship between the strain properties and sequence-dependent seeding barriers in amyloid propagation [167, 168]. It was observed that fibrils generated from human and mouse PrP23–144 have similar conformational properties, while these properties of fibrils formed by Syrian hamster protein are quite different. Human and mouse fibrils could cross seed fibril formation by each other's monomers, but not hamster monomers. Hamster fibrils, on the other hand, could seed the conversion of mouse but not human monomers, reflecting an asymmetric seeding barrier. Importantly, cross-seeding of mouse monomers with hamster amyloid resulted in the emergence of a new strain of mouse fibrils that inherited conformational properties of hamster fibril seeds. Importantly, this new strain of mouse fibrils also lost the original seeding specificity of mouse protein and acquired that of hamster fibrils, indicating that seeding specificity correlates with fibril conformation. These model experiments revealed the mechanism of "conformational adaptability" in cross seeding of PrP amyloid *in vitro* [168], providing strong support for the notion deduced from observations *in vivo* [152, 169] that prion strains and transmissibility barriers are intimately related phenomena, resulting from the ability of prion protein to adopt multiple aggregate conformations. The picture emerging from all these studies is that, while a large spectrum of conformations may be possible among PrP from different species, amino acid sequence variability dictates that only a subset of them is thermodynamically accessible in a given species. If a conformation of a specific donor strain overlaps with the spectrum of conformers accessible to the prion protein of the host, transmission (or seeding in the case of *in vitro* studies) will occur, and this will happen regardless whether the donor and acceptor represent the same or different species. However, if the conformation of the donor strain is outside the spectrum of conformers accessible to the host protein, a barrier to transmission (seeding) will be observed.

While this conformational model of prion strains and transmissibility barriers has become increasingly accepted, what remains perplexing is the large number of prion strains observed *in vivo*. For example, it appears that in mice alone more than

15 distinct strains can be faithfully propagated [170]. How can a protein adopt so many different conformations? A possible structural explanation of this phenomenon has been recently suggested based on crystal structures of small segments taken from amyloid forming proteins. These structures have revealed a common motif called a steric zipper characterized by the segments being arranged in β-sheets whose side-chains form a tight zipper-like interaction along a self-complementary interface [149, 150]. Furthermore, it has been shown that the same segment can form multiple polymorphic steric zipper structures [123]. These structures have been used to illustrate how amyloid strains can arise from a single polypeptide as the result of polymorphic steric zippers. Four types of polymorphisms, "packing," "segmental," "combinatorial," and "single-chain registration," have been hypothesized (Fig. 7) [123], and from this it can be inferred that various combinations of these can give rise to a large number of stable amyloid conformations, each one capable of encoding for a different strain. In this way, the theoretical number of prion strains may even far surpass the number of those which have so far been documented experimentally.

7 Generation of Prion Infectivity *In Vitro* and Synthetic Mammalian Prions

The general concept that proteins can be infectious has been formally proven in studies on protein-based inheritance in yeast and other fungi. In the latter case, the prion state results from the self-association of specific fungal proteins (all of which are unrelated to PrP with respect to both sequence and structure) into amyloid fibrils that self-propagate by a mechanism involving seeding and fibril fragmentation, while transmission occurs naturally through cell division [2, 92, 171]. Transfection of amyloid fibrils generated *in vitro* from bacterially expressed recombinant yeast prion proteins into living cells was shown to result in inheritable prion states that faithfully propagated conformation-encoded strain properties, providing a "proof-of-principle" for the protein-only hypothesis [107, 108]. The path towards understanding the molecular basis of mammalian prion infectivity has proven to be more elusive, though remarkable advances have been made in the past few years.

Early efforts to recapitulate PrP^{Sc} formation and mammalian prion propagation *in vitro* included cell-free conversion reactions in which PrP^{C} was incubated in the presence of PrP^{Sc} from TSE-affected animals [85, 172]. Such simple incubation resulted in species- and strain-specific conversion of substrate PrP^{C} to a PrP^{Sc}-like conformation (as judged by very similar PK resistance) [172]. The yields of these conversion reactions were, however, very low, typically substoichiometric with respect to the input PrP^{Sc} template. Furthermore, no infectivity could be attributed to the newly converted material [173], indicating that not every PK resistant form of the prion protein is necessarily associated with prion infectivity.

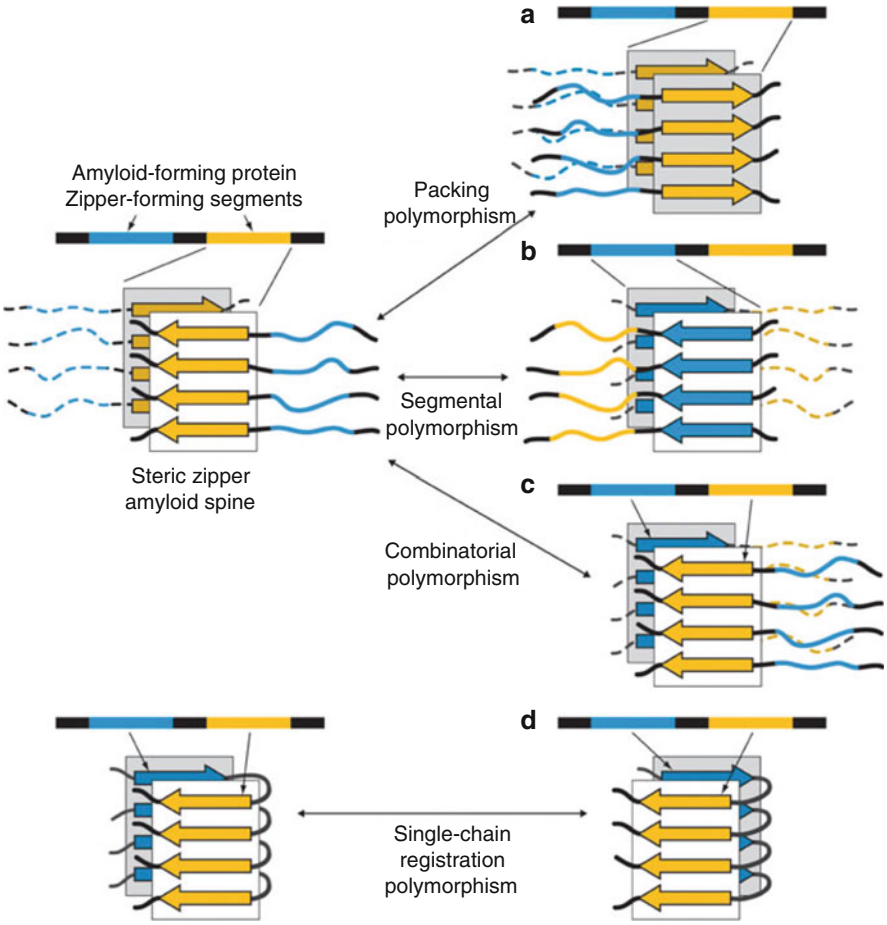

Fig. 7 A possible explanation of prion strain diversity based on the polymorphism of steric zipper motifs observed in crystal structures of short amyloidogenic segments. (**a**) Identical amyloidogenic segments have been found to form distinct steric zipper structures with different self-complementary packing arrangements. This type of "packing polymorphism" predicts that a protein could achieve a number of amyloid structures by oligomerizing along the same segment with different interfaces. (**b**) Because proteins possess multiple steric zipper segments, "segmental polymorphisms" predict that a protein can oligomerize along different segments. (**c**) Although not yet observed at an atomic level, "combinatorial polymorphisms" predict that a protein could oligomerize through a mixed steric zipper interaction of two nonidentical segments. (**d**) A "single-chain registration polymorphism" shows a type of packing polymorphism that is possible between two segments on the same polypeptide chain. Such a polymorphism predicts that a protein could from multiple amyloid structures by shifting the registration of the sidechains within its steric zipper interface. This figure was reprinted from [123] with the permission of *Nature Structural and Molecular Biology*

An important technological advance in TSE research is the invention, by Soto and coworkers, of a procedure called PMCA [174, 175]. In this approach, normal brain homogenate containing PrP^{C} is typically mixed with a minute quantity of brain homogenate from TSE-infected animals (or partially purified PrP^{Sc}) and is then subjected to multiple rounds of sonication and incubation, resulting in highly efficient amplification of the PrP^{Sc} conformer. The products are then diluted into additional normal brain homogenate for subsequent serial amplification cycles, allowing total elimination of the original input PrP^{Sc} and other molecules present in the TSE-infected brain homogenate. Castilla et al. showed that inoculation of hamsters with the product of such serial PMCA results in a classical scrapie disease indistinguishable from that caused by brain-derived scrapie prions, providing the first conclusive evidence that TSE infectivity can be amplified in a cell-free system [175]. This remarkable finding was corroborated in subsequent experiments with mouse prions, and has demonstrated that the material amplified by PMCA faithfully replicates the strain properties of parent prions [176]. Furthermore, a modified PMCA protocol was successfully used to generate infectious prions *de novo* (i.e., from normal brain homogenate alone, in the absence of any pre-existing PrP^{Sc} seeds) [177], providing a potentially valuable model for studying the origins of sporadic TSE diseases. The generation of infectious material by PMCA represents a very important development, strongly supporting the prion hypothesis of TSE diseases. However, the presence of many molecules in brain homogenate used as a substrate makes it difficult to determine the precise molecular composition of the prion agent.

To overcome this difficulty, Supattapone and coworkers applied the PMCA procedure to a simple system consisting of PrP^{C} partially purified from normal hamster brain as a substrate and scrapie PrP^{Sc} (PrP27–30) as a seed [178]. Attempts to propagate TSE infectivity using these components alone were unsuccessful. However, infectious material (with relatively high infectivity titer in hamsters) was generated upon supplementing the PrP^{C} substrate with synthetic poly(A) RNA [178]. Remarkably, the authors reported that TSE infectivity could also be generated from the mixture of PrP^{C} and poly(A) RNA spontaneously, i.e., without requiring seeding with brain-derived PrP^{Sc}. These findings represent a major step towards defining the chemical composition of mammalian prions, indicating that polyanions such as RNA and fatty acids that co-purify with PrP^{C} are potentially crucial cofactors required for efficient conversion of prion protein to the form associated with TSE infectivity. However, it is not clear whether these cofactors act simply as catalysts of $PrP^{C} \rightarrow PrP^{Sc}$ conversion or, rather, represent essential constituents of the infectious prion particles. It is also unclear whether single-stranded nucleic acids are indeed required for prion replication *in vivo*, or are merely surrogates that mimic the action of other polyanionic molecules such as, for example, sulfated glycosaminoglycans [179].

Another line of studies involves experiments with bacterially expressed rPrP. Such studies are important since they can eliminate any potential ambiguities with regard to the presence of even minute quantities of mammalian cofactors that may co-purify with brain-derived PrP^{C}. In the first study of this type, Legname et al. used

amyloid fibrils formed by recombinant mouse prion protein fragment 89–230 [180]. Intracerebral injection of these fibrils into transgenic mice expressing the same N-truncated form of PrP resulted in a TSE disease, though only after very long incubation times (380–660 days after inoculation). These experiments were repeated using another line of transgenic mice (this time expressing full-length PrP^C) and different types of rPrP amyloids with varying conformational stabilities, finding an intriguing correlation between amyloid stability and incubation periods required to produce clinical symptoms [181]. The authors concluded that their results establish "beyond reasonable doubt" that infectious mammalian prions are composed solely of the misfolded form of PrP. However, as pointed out by many commentators [114, 152, 182], the interpretation of these strikingly interesting experiments with "synthetic prions" is clouded by the fact that the transgenic mice overexpressed the prion protein (at a level 4–32 times that of wild-type animals), raising questions as to whether the inoculated fibrils were *bona fide* infectious or, rather, accelerated a condition to which these mice could be predisposed due to a high level of PrP^C. Another puzzle of these studies relates to the very long incubation periods that were observed, especially when considering the large quantities of the material used to inoculate the animals. While this could reflect the unusual strain properties of synthetic prions, another possibility is that this effect is due to a very low infectivity titer of amyloid preparations, implying that only a minute fraction of this material is associated with prion infectivity. It has also been proposed that amyloid preparations represent conformational intermediates along the folding pathway from PrP^C to PrP^{Sc}, which require maturation to generate specific prion strains following inoculation [181]. However, in the latter scenario, the infectivity would be generated *in vivo* rather than *in vitro*, and this process could involve the recruitment of cellular cofactors.

In a continued quest to generate synthetic prions that cause TSE disease in wild-type animals, Makarava et al. used rPrP amyloid fibrils that were "annealed" by incubation at low temperature in the presence of normal brain homogenate [140]. In contrast to non-annealed fibrils that are characterized by a relatively short PK-resistant core, a small proportion of annealed material showed PK-resistance similar to that of brain-derived PrP^{Sc}. Upon inoculation of these fibrils into Syrian hamsters, none of the animals developed clinical symptoms, but a fraction of them showed detectable levels of brain PrP^{Sc}. In second passage experiments using brain homogenate from these PrP^{Sc}-positive hamsters as the inoculum, all hamsters developed a TSE disease characterized by unique symptoms and very long incubation periods. Though the mechanism of fibril annealing and the identity of specific cofactors involved remain unknown, these studies provide experimental evidence that the material generated *in vitro* from rPrP can induce transmissible prion disease in wild-type animals.

While the PMCA technology proved so powerful in generating infectious material from brain-derived PrP^C [175], early attempts to use this method with rPrP were unsuccessful. In fact, rPrP was shown to inhibit the conversion of PrP^C using the standard PMCA protocol [183, 184]. More recently, however, Wang et al. developed a protocol allowing *de novo* generation of bona fide infectious prions by

PMCA from a chemically well-defined mixture consisting of the recombinant mouse PrP, mouse liver RNA, and a synthetic acidic phospholipid, 1-palmitoyl-2-oleoyl-phosphatidylglycerol [185]. Remarkably, these synthetic prions were infectious to wild-type mice, leading to clinical TSE disease ~130 days after inoculation. Such short incubation period strongly suggests the high infectivity titer of this material. The apparent requirement for polyanions and lipids for infectivity of these PMCA-generated rPrP prions is reminiscent of the previous finding on prion generation from brain-derived PrP^{C} [178], again pointing to an important role of these cofactors. However, in yet another study, Kim et al. show that prion infectivity can be generated by a modified PrP^{Sc}-seeded serial PMCA procedure solely from recombinant hamster PrP, in the absence of any added cofactors such as polyanions or lipids [186]. These apparently purely proteinaceous prions, generated in a seeded PMCA reaction, were infectious to wild-type hamsters, though their infectivity titer was low as indicated by relatively long incubation periods and an incomplete attack rate. These recent studies leave little doubt that mammalian prions can be generated *in vitro* from bacterially expressed rPrP. However, the role of specific cofactors in this process remains controversial. Are these cofactors absolutely necessary components of prion infectivity or, rather, do they act as nonobligatory catalysts that merely enhance prion protein misfolding to the infectious form?

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Molecular Dynamics as an Approach to Study Prion Protein Misfolding and the Effect of Pathogenic Mutations

Marc W. van der Kamp and Valerie Daggett

Abstract Computer simulation of protein dynamics offers unique high-resolution information that complements experiment. Using experimentally derived structures of the natively folded prion protein (PrP), physically realistic dynamics and conformational changes can be simulated, including the initial steps of misfolding. By introducing mutations in silico, the effect of pathogenic mutations on PrP conformation and dynamics can be assessed. Here, we briefly introduce molecular dynamics methods and review the application of molecular dynamics simulations to obtain insight into various aspects of the PrP, including the mechanism of misfolding, the response to changes in the environment, and the influence of disease-related mutations.

Keywords Prion protein · Molecular dynamics simulation · Protein dynamics · Protein misfolding · pH-induced misfolding · Disease-related mutations

Contents

M.W. van der Kamp and V. Daggett (✉)
Department of Bioengineering, University of Washington, Box 355013, Seattle, WA 98195–5013, USA
e-mail: daggett@u.washington.edu

1 Introduction

Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative diseases that occur in mammalian species. They include scrapie in sheep, bovine spongiform encephalopathy in cattle, chronic wasting disease in deer and elk, and Creutzfeldt–Jakob disease (CJD) in humans. These prion diseases can arise spontaneously as a rare "sporadic" disorder, caused by hereditary or somatic mutations, or through infectious transmission. The notion that the infectious disease agent in TSE could be devoid of nucleic acids and primarily exists of protein, the so-called protein-only hypothesis, was first advanced in the 1960s based on experimental observations [1] and theory [2]. Prusiner and colleagues later showed that a particular protein was indeed required for infectivity [3–5]. Based on the name given to such a protein-based nucleic-acid free agent, a *pro*teinaceous *in*fectious particle or *prion*, the protein was called the prion protein (PrP). Further pathological studies showed that the typical, often fibrillar, amyloid deposits, found in the brains of inoculated individuals, contained host-encoded PrP [6]. Together, these findings sparked wide-ranging studies on PrP, both in vivo and in vitro.

The benign and natively folded cellular form, PrP^{C}, was isolated and characterized in detail. It is largely soluble and has high α-helical content with little β sheet [7–9] (see Sect. 3.1). In vivo, it is primarily found attached to the outer cell membrane of neuronal cells [10], via a glycosylphosphatidyl-inositol (GPI) anchor linked to the protein C-terminus [11] (Fig. 1a). Determining the function of PrP^{C} has proved to be a major challenge, complicated by the fact that PrP knock-out mice lack an obvious phenotype [12]. Many different putative functions have been proposed, indicating that PrP^{C} is a multifunctional protein that plays a role in cell signaling [13, 14] and metal metabolism [15–17]. When PrP aggregates and forms fibrils, however, it has significantly changed conformation and becomes largely insoluble and proteinase K resistant. It is likely that early, non-fibrillar aggregates represent the infectious particles and cause neurotoxicity [18]. Together, the various aggregates consisting of misfolded PrP are often denoted PrP^{Sc} for scrapie. Apart from the fact that PrP^{Sc} has a significantly increased β-sheet content and decreased α-helical content [7, 19, 20], little is known about its precise conformation from experiment. The conversion from PrP^{C} to PrP^{Sc} appears to be triggered by a decrease in pH [21, 22], introduction of mutations [23, 24], and by the presence of PrP^{Sc} [25].

Despite the continuing research into various aspects of the PrP, many open questions remain. These include the precise function of PrP^{C}, the mechanism of PrP^{C} to PrP^{Sc} conversion, the nature of the infectious and neurotoxic particles, and the mechanism of neurotoxicity. Current research efforts therefore cover many different

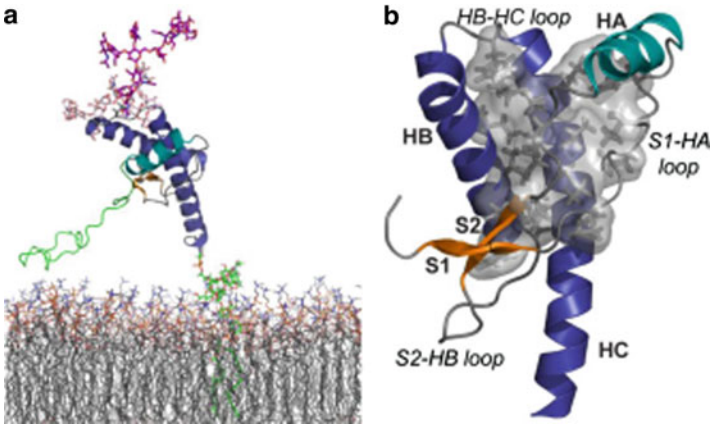

Fig. 1 Structure of the human prion protein. (**a**) The mature human prion protein as it can be found on the outer cell membrane. The GPI-anchor (attached to Ser230) and typical glycans (attached to Asn181 and Asn197) are shown in *sticks*. Structure obtained from MD simulation (Van der Kamp, Koldsø and Daggett, unpublished results). (**b**) The structured part of the recombinant human prion protein, as obtained by protein NMR [39]. Secondary structure elements are labeled

aspects and employ a wide variety of experimental methods, from structural biology to in vivo studies. Computational studies can provide a complementary tool to help elucidate some of the outstanding questions. In the last decade, computer simulation of biomolecules, in particular proteins, has advanced significantly [26]. A method that has had a particularly large impact is molecular dynamics (MD) simulation. This technique allows for the detailed examination of the complex internal motions and conformational changes in proteins, which is often important for understanding their function [27–29]. Furthermore, MD simulations provide uniquely detailed information necessary to understand protein folding [30, 31] and, crucially, disease-related protein misfolding [32, 33]. Accurate all-atom MD simulations have now been performed on a large scale, across essentially all known protein folds, opening the way to obtain fundamental insights into protein dynamics and folding [34].

MD simulation was first used in PrP research to study the conformational preferences of a small fragment, indicating how a disease-related mutation may favor aggregation [35]. Soon after detailed structural information became available [8, 36–39], MD simulation was employed to study the folded domain of PrP [40–43]. These initial studies were limited, allowing for studying local dynamical effects only. Currently, advances in computer power and algorithms provide the means to perform multiple simulations of tens to hundreds of nanoseconds. Although this may still be short in terms of biological timescales, the more extensive simulations make detailed comparisons to experiment possible. Furthermore, longer simulations are able to capture significant conformational changes, such as those involved in misfolding.

In this chapter, we start with a brief outline of the theoretical aspects of MD simulations. Then we review how these methods have been used to explore the

dynamics and misfolding of the PrP, and how this information was used to suggest models for early aggregates. Thereafter, we highlight applications of MD simulations that provide insight into the effects of mutations related to human prion disease. We then briefly describe other aspects of PrP that have been studied using MD, such as the influence of post-translational modifications and small molecules. We close with an outlook of how MD studies can further increase our knowledge of PrP in the future.

2 Molecular Dynamics Simulation

MD Simulation of proteins at the atomic level is a well-established technique [29, 44, 45]. By explicitly representing all atoms and bonds in a macromolecule, it provides physically realistic information on how this molecule, e.g., a protein, evolves over time. The resulting "trajectory" is recorded at high temporal resolution and can be analyzed using a wide range of techniques, offering uniquely detailed insight into local dynamics, stability, flexibility, and possible conformational changes in proteins.

2.1 Principles

A typical protein contains hundreds to thousands of atoms and therefore has an even larger number of degrees of freedom. When the solvent around a protein is considered explicitly, the number of atoms and degrees of freedom increases even further. In order to model such a complex system efficiently, electrons are generally ignored and properties of the system are calculated based on the nuclear positions only. In combination with a potential energy function that describes electronic phenomena such as chemical bonding, spatial configurations of atoms, and electrostatic interactions, this simplification allows the use of classical mechanics to describe the system. This type of modeling is generally described as *molecular mechanics* [46]. The potential energy function and the parameters for the different atoms and configurations of atoms used in this function are called a *force field*. Current force fields optimized for proteins are well established and describe protein dynamics with similar accuracy [47]. They use similar potential energy functions, in which, for example, bonds and angles are represented by harmonic terms, electrostatic interactions are described by atomic partial charges and the Coulomb equation, and Van der Waals forces are included through a simple Lennard-Jones function [48, 49].

The force field defines the energy of a particular atomic configuration. In order to describe the dynamics of a protein system, it is necessary to (1) get a starting configuration of the atoms in the system, (2) set this configuration in motion, and (3) calculate a new configuration based on that motion. High-resolution starting

configurations for many proteins can be obtained from the Protein Data Bank (PDB) [50], the depository of protein structures usually determined by X-ray crystallography or protein nuclear magnetic resonance (NMR) techniques. Not every structure in this database will be of high enough quality for simulation. Also, in many cases, positions for missing atoms will need to be assigned, e.g., hydrogen atoms (not observed in X-ray crystallography) and atoms in parts of the structure that are too flexible to be determined in the experiment. Once a starting conformation is obtained, and (in the case of simulation with explicit solvent) solvent molecules are added in optimized positions, the system can be set in motion. In order to do so, velocities are assigned randomly, typically restrained by the Maxwell–Boltzmann distribution at a chosen temperature. Given these initial velocities and atomic masses, the forces on all atoms in the systems are now described by the derivative of the potential energy defined by the force field. These forces can in turn be used to calculate a new set of atomic positions and velocities. In order to obtain a physically accurate new atomic configuration, the time step (the amount of time between one configuration and the next) must typically be $\leq$2 fs (smaller than the fastest movement in the system, e.g., bond vibration). In principle, MD simulation is a deterministic technique: given one set of atom positions and velocities, one series of configurations through time, or a trajectory, will be the result.

2.2 Scope, Limitations, and Variations

Within the limitations of the accuracy of force fields, MD simulation predicts the motion of a molecular system through time. The simulation lengths that can be assessed have greatly increased by the developments in computer hardware and efficient algorithms. However, current simulations are typically still limited to tens to hundreds of nanoseconds, whereas many biological processes occur on much longer timescales. The combination of the high temporal and high spatial resolution obtained in MD simulations, however, is unattainable by experimental techniques. One could see an MD simulation as a "computational experiment" that can reveal the motion of biomolecules in great detail. Just as in lab experiments, it is important to repeat the experiment (i.e., the simulation) to substantiate any conclusions drawn. Now computational resources allow one to do so, it is good practice to perform several simulations of the same system, starting from different initial velocities.

Another way to view MD simulation is as a technique to probe the atomic positions and momenta that are available to a molecular system under certain conditions. In other words, MD is a statistical mechanics method that can be used to obtain a set of configurations distributed according to a certain statistical ensemble. The natural ensemble for MD simulation is the microcanonical ensemble, where the total energy E, volume V, and amount of particles N (NVE) are constant. Modifications of the integration algorithm also allow for the sampling of other ensembles, such as the canonical ensemble (NVT) with constant temperature

(T) instead of constant energy, or in the isothermal-isobaric ensemble (NPT) in which pressure is constant instead of volume. The structural configurations of a protein that are accessible within these conditions are governed by the free energy landscape. When standard all-atom explicit solvent MD simulation is used, physically realistic conformational transitions between such configurations are sampled. The nature of the free energy landscape, however, reduces the likelihood of sampling rare transitions (such as misfolding). Given the limitations in timescale, the system is more likely to sample conformations within a set of closely related local minima, a "valley" within the free energy landscape. There are several ways in which more comprehensive sampling of conformational space can be achieved, although this usually comes at the cost of representing a physically relevant trajectory or pathway. A simple way to increase sampling is to raise the temperature used in simulation. This effectively increases the energy available to the system to overcome free energy barriers. Another technique using this principle is replica-exchange MD [51]: several noninteracting replicas of the simulation system are run at different temperatures, and the replicas are allowed to exchange with one another when similar conformations are sampled. Another technique that helps to avoid the multiple minima in the free energy landscape is *metadynamics*, aimed at avoiding minima that have already been sampled within a trajectory [52]. However, these two methods cannot provide pathways for a process, or the mechanism of a conformational change. Instead they are used for sampling different states. In order to enhance the timescales accessible by MD simulation, the number of particles to consider can be reduced by the use of implicit solvent methods or a *coarse-grained* representation of the system, in which multiple atoms (e.g., in an amino acid side chain) are treated as one particle.

3 The Dynamics and Misfolding of the Wild-Type PrP

MD simulations have been employed extensively to study the conformational dynamics of the wild-type PrP for a range of species. A wide variety of force fields, setups, and analyses has been used to this end. The majority of studies is performed on human, mouse, or hamster PrP. In all but a few contributions [53–55], simulations include only the protein portion, i.e., the unglycosylated form without membrane anchoring. These simulations therefore present the recombinant PrP (recPrP) that is used for many in vitro studies, usually produced in *Escherichia coli*. Often, only the structured part of recPrP is simulated. Although most initial MD studies could only capture local dynamics [40–43], some studies were able to shed light on the misfolding process over short timescales (~10 ns) [56, 57]. More recent studies reporting multiple simulations of 50 ns or more [58, 59], have provided a more comprehensive view of the conformational dynamics.

Apart from studying the native dynamics of recPrP in solution, MD simulation has also been used to sample possible early events in PrP misfolding, either by simulating under conditions that are known to promote misfolding, such as low pH

[56, 57, 59–61], or by using enhanced, nonphysical sampling methods [62, 63]. As details of the misfolding pathway are unknown and difficult to probe by experiment, the results obtained can only suggest potential events and conformations along the pathway. Careful comparison with available experimental data, however, may validate the simulation results.

3.1 The Starting Point: PrP Structures from Experiment

High-resolution structural information is required as a starting point for atomistic MD simulations. For PrP, the first [8] and most abundant structural information has been obtained by NMR techniques, which has resulted in experimentally derived models of PrP of a wide variety of species [64–70]. For human and sheep PrP, structures determined by X-ray crystallography have also been reported, both with and without antibodies bound [71–75]. The overall structure of PrP that has emerged from these studies is largely identical for the different species and conditions. The mature protein (residues 23–230 in human PrP numbering, used throughout in this chapter) exhibits a highly flexible N-terminal domain, consisting of the first ~100 residues, and a folded or globular C-terminal domain, spanning residues 125–228. The final residues (229–230) also appear to be flexible.

The globular domain contains a short β-sheet, existing of two strands (S1 and S2 with res. 128–131 and 161–164, respectively), and three α-helices (HA, HB, and HC) (Fig. 1b). The first α-helix, HA, is the shortest. It spans residues 144–156, with the last three residues forming a 3_{10} helix at neutral pH and a less regular structure at pH 4.5 [39, 76]. The second and third α-helices, HB (res. 172–194) and HC (res. 200–228), are connected by a disulfide bond: Cys179–Cys214. This disulfide bond is retained when PrP misfolds and aggregates [77, 78]. Around it, there are several hydrophobic residues that link HB and HC together and form a stable core of the protein [79]. The C-terminal end of HB (res. 187–194) is significantly less stable than the rest; NMR studies reveal that it can exist in a disordered conformation [39] and hydrogen-exchange protection of the backbone amides is low [39, 76, 79]. The many threonine residues in this sequence (HTVTTTTK, conserved in mammalian PrPs) cause this part of HB to have an inherently low helical propensity [80]. Notably, this part of HB appears to be stabilized to some degree by the presence of the flexible N-terminus, likely due to contacts [39]. At the top of HC, a so-called "capping box" interaction [81] may help stabilize the helical structure [82]. For the C-terminal portion of HC, there are also indications that its conformation can be flexible. Residues 220–231 are considered partly disordered in mouse PrP [8] and human PrP with the R220K mutation [83]. NMR relaxation studies on hamster and mouse PrP further indicate fast picosecond timescale motions of the backbone amides from residue 222 [84, 85].

The detailed structural information obtained from experiment for the globular domain of PrP^C provides a starting point to perform MD simulations. One must realize, however, that uncertainties in the structure of PrP in aqueous solution still exist. Using protein NMR techniques, a set of conformational constraints is

obtained that is subsequently used to generate likely structural models that satisfy these constraints [86]. Although this method generally provides a good description of the overall fold, the positions of certain residues or side-chains may be less well-defined. X-ray crystallography has similar limitations, although high-resolution electron density maps offer more certainty. The crystallization process may, however, influence the protein conformation, e.g., through crystal packing, dimerization, and domain swapping. The structure obtained may therefore not be fully representative for the conformation in solution.

3.2 The Influence of pH on PrP Dynamics and Conformation

A range of experiments have indicated a relationship between a decrease in pH and misfolding and aggregation of PrP [87]. In human recPrP, significant conformational changes were observed between pH 6 and pH 4.4 [22], involving exposure of hydrophobic surfaces, thereby facilitating aggregation. Human PrP extracted from brain cells forms detergent-insoluble aggregates at pH 3.5 and 1.5 M guanidinium chloride [88]. The initial changes are accompanied by a decrease in thermodynamic stability of recPrP relative to neutral pH, as evidenced by continuous-flow fluorescence [89] and NMR [76]. Further, studies of human recPrP under acidic and mildly denaturing conditions suggest the presence of intermediate states during misfolding and oligomerization: a native-like α-helical conformation was observed at pH 4.1 and a conformation with β-sheet characteristics at pH 3.6 [90]. For mouse and hamster recPrP, similar changes were observed in response to a decrease in pH [91, 92]. In particular, loosening of the tertiary structure of hamster recPrP occurred below a pH of 4.7, with a minor shift to β structure at pH 4.0. This was accompanied by a decrease in binding of antibodies to epitopes in the flexible N-terminus [92], indicating that this flexible tail changes during misfolding. More recently, spontaneous aggregation and fibril formation of human recPrP was achieved under conditions of pH 4.0 and slow rotation, without addition of denaturants [21]. Altogether, it is evident that pH affects conformation and stability of PrP, and acidic pH can cause misfolding and aggregation. This is relevant for the occurrence of misfolding and aggregation in vivo, as it has been suggested that these processes may take place in endosomal compartments [93–96], which are mildly acidic [97].

In standard molecular mechanics methods, all atoms and bonds between atoms are explicitly defined, i.e., they are either present or not. In order to model the changes in pH, one must therefore alter the protonation states of ionizable amino acid side chains. For a decrease in pH, the relevant side chains are those of histidine, glutamate, and aspartate. Their pK_a values in solution are 6.08, 4.15, and 3.71, respectively [98]. The local protein environment can, however, change the pK_a of individual residues significantly. Langella et al. [99] calculated theoretical pK_a values of the relevant side-chains based on the coordinates deposited for human recPrP obtained by protein NMR at pH 4.5 and pH 7.0 [39, 76]. Although differences in the local conformation of residues between the various structures lead to a

range of pK_a values, their results suggest that two of the four histidine residues in the globular domain (His140 and His177) will be protonated at very mildly acidic pH (pH $<$ 6.5). The other two (His155 and His187), however, may only become fully protonated around pH 4.5. At this mildly acidic pH, solvent-exposed glutamate residues may also become protonated and a further decrease to strongly acidic pH (pH $\leq$ 3.0) will likely result in protonation of all glutamate and aspartate side chains. In line with these values, several MD studies have used differential protonation of side chains to compare the conformational dynamics of human PrP between neutral and strongly acidic pH [56, 57, 61], neutral and mildly acidic pH [99, 100], and all three pH environments [59, 101]. The neutral pH environment was represented by using singly protonated (neutral) histidine side chains and all other ionizable side chains charged, mildly acidic pH by all ionizable side chains charged, and strongly acidic pH by all ionizable side chains protonated. One study also simulated species with only part of the histidine side chains charged [99]. For hamster and bovine PrP, MD simulations have also been performed in a strongly acidic pH environment [56, 57], predominantly to capture the process of PrP misfolding (see further details in Sect. 3.3).

All studies on the effect of pH on WT PrP published before 2007 reported single MD simulations, mostly of 10 ns. In 2007, DeMarco and Daggett reported three simulations of 15 ns of human PrP at both neutral and strongly acidic pH [61]. As in all previous studies, the overall fold was stable at neutral pH. Significant changes were observed for one simulation at strongly acidic pH, which was linked to misfolding of the protein (see further below). Recently, we performed the most extensive MD simulations of the effect of pH on human PrP to date, including detailed comparisons to experimental data [59]. In this study, five simulations of 50 ns at each of the three pH environments were compared. In the remainder of this section, we will focus on this study to highlight the information obtained by MD simulation on the effect of pH on the structure and dynamics of PrP.

At neutral pH, the overall fold is stable, without significant changes in secondary or tertiary structure. The flexibility of the S2-HB and HB-HC loops is high, in accordance with experimental data [84, 85]. Although the capping box at the N-terminal end of HC was not present in the starting structure (obtained from the representative NMR structure with PDB code 1QLX), it formed within 1 ns in all simulations at neutral pH. Also, a hydrogen bond between His187 and the main-chain carbonyl of Arg156, which anchors HA to HB, was present for ~30% of the simulation time. Comparisons with publicly available distance restraints obtained from NMR further indicated that relevant conformational ensembles were sampled and maintained throughout all five runs.

Theoretical pK_a calculations indicate that the first two histidine side chains to become protonated are His140 and His177. In a single 10 ns simulation, Langella et al. found little difference from a neutral pH simulation [99], as could be expected from their initial placement out into solvent. With all histidine side chains protonated, however, simulations revealed more significant changes [59]. In this mildly acidic pH environment, the 3_{10}-helix conformation in res. 153–156 that was formed for ~25% of the time at neutral pH largely disappeared. This is in agreement with the

NMR studies and the simulations indicate that this change is due to a repulsion between the side chains of His155 (now protonated) and Arg156. A more significant change, which was not directly apparent from the NMR structure obtained at pH 4.5, was observed in the loop between HB and HC. Phe198, central in this loop and part of the hydrophobic core at neutral pH, moved out into solvent. This change was accompanied by a change in the loop conformation and disruption of the capping box on top of HC. These changes may be related to the protonation of His187, the histidine with the lowest theoretical pK_a value (and therefore perhaps largely unprotonated in the NMR experiment). In the simulations at mildly acidic pH, a salt bridge interaction between His187 and Asp202, involved in the capping box at neutral pH, is formed.

The strongly acidic environment introduces significant changes in the PrP structure: protonation of the 5 Asp and 9 Glu side chains in the globular domain causes the overall atomic charge in this domain to rise by 14 a.u. compared with the mildly acidic regime. A large effect on the conformation and dynamics can therefore be expected. Notably, changes to the most stable part of the PrP structure (as determined by NMR and hydrogen exchange [39, 76, 79, 85]) are minimal (Fig. 2). A major change

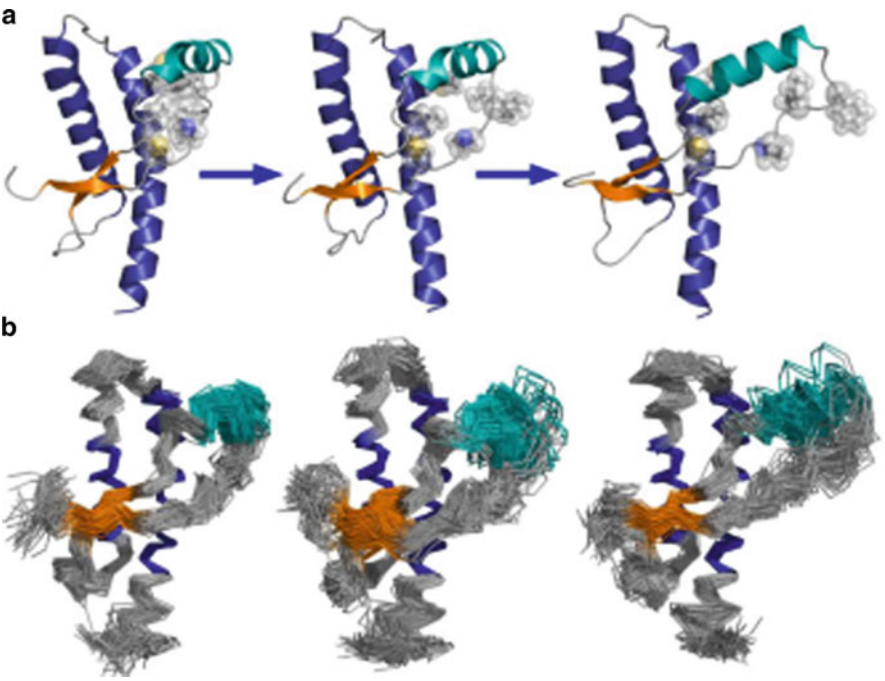

Fig. 2 Conformational changes in the globular domain of PrP induced by acidic pH [59]. (**a**) Snapshots from a simulation at acidic pH, indicating the loss of hydrophobic contacts between the S1-HA loop and HC, followed by displacement of the N-terminal end of HA. (**b**) Cα traces of simulations at neutral, mildly acidic and strongly acidic pH from *left* to *right*. Structures of five simulations at each pH regime are shown for every 1 ns in the 25–50 ns time interval. The parts of HB and HC that remain stable (in agreement with experiment) are indicated by *darker colors*. In both panels, N-terminal residues 90–124 are omitted for clarity

observed in three of the five simulations, also found in several other studies [56, 57, 61, 102], is a repositioning of HA. The N-terminal part of helix swings away from the stable HB-HC core, out into solvent. Interestingly, this part was determined to be the most pH sensitive site in cysteine-scanning spin-labeling ESR studies [103]. Detailed analysis indicates that this movement is always preceded by a decrease in contacts between the hydrophobic residues in the S1-HA loop and those on HC (Fig. 2). Interestingly, unfolding studies also indicate that the S1-HA loop and HA can rearrange and become detached from the remainder of the protein [104, 105]. The changes in the S1-HA loop and HA may therefore be related to misfolding.

3.3 Misfolding and Aggregation

Due to the importance of PrP misfolding for development of TSE diseases and the technical difficulties involved in probing this process experimentally, many MD studies have aimed at probing the initial events in conversion of PrP^{C} to PrP^{Sc}. Different strategies have been used to increase the likelihood of observing such initial misfolding when starting from the native PrP^{C} structure, which should be a rare event. One strategy is the introduction of single-residue mutations that have been shown to destabilize the native PrP^{C} fold and promote aggregation. For example, Hirschberger et al. studied the M205R and M205S mutations in the structured part of human PrP (res. 125–228) in single 10 ns MD simulations [106]. Cell studies had indicated that these mutations interfere with folding and can adopt a misfolded conformation [107]. In simulation, the native fold was indeed destabilized by both mutations, but in significantly different ways: with M205S unfolding of the central part of HB (res. 181–188) occurred, whereas with M205R, HA moved out to solvent and subsequently lost helical structure. It is not clear if these events are related to the misfolding pathway of WT PrP, because the mutant proteins may never adopt the native fold. In Sect. 4, we will discuss the use of MD to study the effects of single residue mutations further.

To increase conformational sampling, replica-exchange MD simulations were performed by De Simone et al. [63], based on the crystal structure of sheep PrP [73] (res. 125–230). One conformational substate found in the simulations was argued to be a possible intermediate for aggregation. It featured similar changes from the native fold as found in regular MD simulations of human PrP at acidic pH [59, 61]: HA was disconnected from the core of HB and HC and a large hydrophobic surface was exposed. The limitation of this work and other studies [62, 106], however, is that the flexible N-terminus was not included in the simulations. There are many indications from experiment that at least part of this region plays a role in misfolding and aggregation [108–111]. We have therefore always included that part of the flexible N-terminus in our simulations. Further, the relation between acidic pH, a decrease in PrP^{C} stability, and conversion of PrP^{C} to PrP^{Sc}, as outlined above, makes it plausible to use simulations in the strongly acidic environment as the conversion-inducing perturbation.

The first MD study to report on the initial, pH-induced conformational conversion of PrP^C [56] used a starting structure of res. 109–219 from recombinant Syrian hamster PrP determined by NMR [37]. Whereas simulation at neutral pH resulted in a stable 10 ns trajectory, a significant increase in flexibility and conformational changes were observed at strongly acidic pH. Importantly, the native β-sheet extended and additional strands were formed in the N-terminal region. Also, HA and the preceding S1-HA loop became disconnected from the rest of the globular domain. Several residues in the S1-HA loop adopted a β-strand-like structure. Similar structural conversions were later also reported in equivalent simulations and fragments of bovine and human PrP [57] and for a longer fragment of human PrP (res. 90–231) [61]. Recently, it was shown that this type of initial structural conversion can also take place under mildly acidic simulation conditions (only His protonated) [59]. The similarities between simulations of a number of different fragments and different conditions provide credence to the early steps of misfolding observed.

Although the three helices remain largely intact in the simulations of early misfolding, the amount of β-structure increases. An additional β-strand is formed onto the native sheet, usually located in residues 116–122 of the flexible N-terminus. Further strands sometimes form in the remaining part that was included in the simulation. Antibody studies indicate that residues 90–120 undergo a conformational change upon conversion to PrP^{Sc} [112–114] and a range of studies support the involvement of residues in this region in PrP^{Sc} formation [108, 110, 111, 115]. Our misfolding simulations also indicate the formation of an isolated strand in the loop preceding HA (res. 136–140) after hydrophobic contacts with HC are lost. Abalos et al. [108] showed that modifications in this loop, such as sequence scrambling and mutations to alanine, interfered with conversion to PrP^{Sc}. Also, NMR studies indicate that the S1-HA loop changes under high-pressure conditions, including the loss of hydrophobic contacts [116]. These experimental data confirm that the pH- and aggregation-related exposure of hydrophobic surface [22] can arise from a conformational change in the S1-HA loop, as first suggested by MD simulation [56].

In addition to the MD studies mentioned above, simulation of the 109–219 fragment of hamster PrP was also performed with the D147N mutation [117]. This mutation does not significantly destabilize the PrP fold, but it does increase conversion efficiency [23]. A 20-ns MD simulation at neutral and strongly acidic pH revealed structures that deviated significantly from the starting structure. At strongly acidic pH, a conformational state arose that was similar to those observed in pH-induced misfolding simulations of WT PrP [56, 57]. Again, the conformation exhibited a three-stranded sheet (formed by extension of the native sheet) and an isolated strand in residues 135–140 (the S1-HA loop). Using a typical structure from this conformational state as a monomer, an oligomeric structure was built by bringing together exposed hydrophobic residues. This included the docking of the isolated β-strand to the additional β-strand formed from the flexible N-terminus. Further addition of monomers led to a spiral oligomeric structure with a 3_1 symmetry axis and a β-sheet core (Fig. 3a). This "protofibril" could be an early aggregate on the pathway to amyloid formation and/or relevant to infectious and neurotoxic particles. The model fits remarkably well to the electron-microscopy

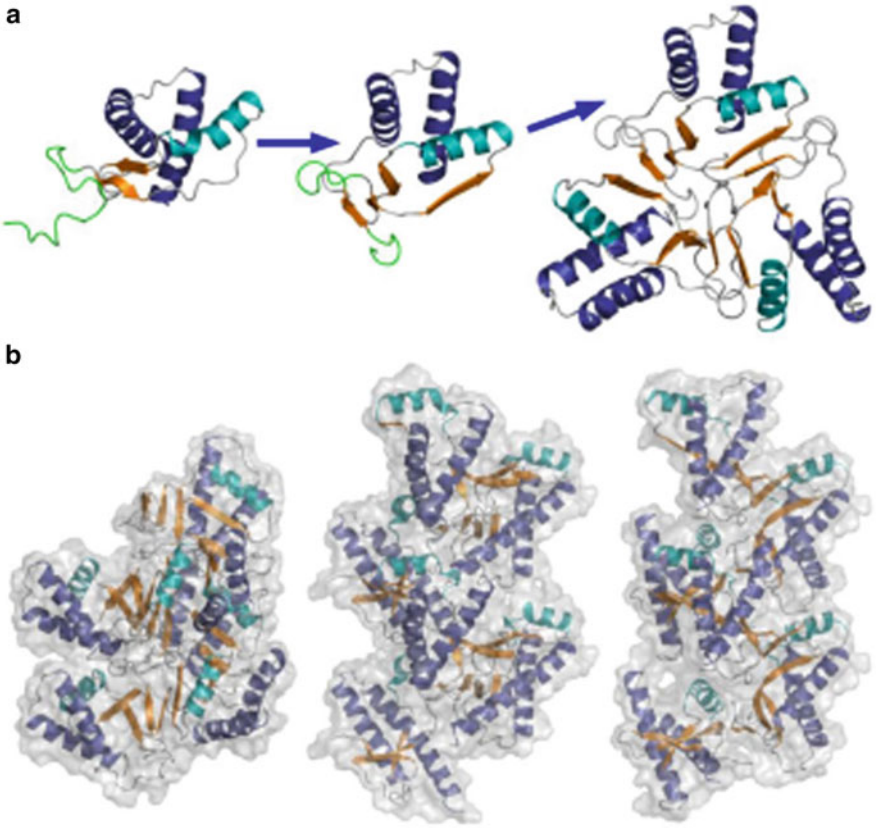

Fig. 3 Potential misfolding and aggregation of PrP. (**a**) Misfolding of D147N hamster PrP observed in simulation at strongly acidic pH and docking of three misfolded monomers into an initial aggregate [117]. (**b**) Spiral protofibril models (built up from six monomers each) for D147N hamster PrP (as shown in panel **a**), bovine PrP and human PrP [121]

data of two-dimensional PrP^{Sc} crystals [118], including the position of res. 142–176 and glycans inferred from electron-microscopy difference maps of PrP27-30 and $PrP^{Sc}106$ [117]. Subsequently, the compatibility between the protofibril model and a range of experimental data further indicated the relevance of this model [119], in contrast to a previously proposed fibril model based on a β-helix structure [120].

After our protofibril model was shown to be plausible, similar models were built for WT hamster, human, and bovine PrP [121]. Again, the initial monomer structure was obtained from MD simulation at the strongly acidic pH regime. In contrast to hamster PrP, human and bovine PrP showed a left-handed spiral formation (Fig. 3b). This difference and further subtle differences between the individual protofibril models may reflect strain differences and give clues to the origin of observed "species barriers" [122]: transmission of prion disease between different species can be inefficient or even absent. Furthermore, the models may help to

rationalize the perceived importance of the S2-HB loop conformation regarding cross-species infectivity [9, 65, 66, 69], as this loop forms a crucial contact area between multiple monomers in the protofibril models.

4 The Effect of Pathogenic Mutations

About 10–15% of prion diseases in humans are caused by mutations in the *PRNP* gene. Three different types of pathogenic mutations exist: premature stop codons, insertion of additional octapeptide repeats in the flexible N-terminus, and point-mutations leading to single amino acid replacements. The latter type of pathogenic mutation has been found in at least 28 different locations, and effects on protein stability, misfolding, as well as cellular processing and function are reported [123]. MD simulations can be used to study the effect of these mutations on protein conformation and stability, particularly for the 24 mutations that are found in the C-terminal folded domain of human PrP [124–126]. Structural biology can also offer insights into the structural effect of pathogenic mutations [75, 127]. In some cases, however, structural studies do not reveal differences between wild-type and mutant proteins whereas MD simulations do (see e.g., [128–130]).

4.1 D178N

The D178N mutation in human PrP is involved in familial prion disease [131]. Intriguingly, the phenotype of disease is significantly altered by the common M/V polymorphism at res. 129: fatal familial insomnia (FFI) arises in combination with M129 and familial CJD arises in combination with V129 [132], although this distinction may not be so clear cut [133, 134]. The aggregation propensity of PrP is significantly increased by the D178 to N substitution. When producing mutant human recPrP (res. 90–231) in *E. coli*, the D178N/M129 mutant aggregated into inclusion bodies [135], as was the case for D178N/M129 and D178N/V129 mouse recPrP [136]. Urea-induced unfolding studies indicated that the thermodynamic stability of PrP decreases by ~22 kJ mol^{-1} upon introduction of the mutation [136]. It was further found that the structure of D178N PrP^{Sc} is different from WT PrP^{Sc} obtained under the same conditions [137, 138].

The D178 side chain can form a salt bridge with R164 (located on the second native β-strand, S2) and may be involved in hydrogen bond interactions with the Y128 and Y169 side chains (located at the beginning of β-strand S1 and the loop between S2 and HB respectively) [56]. The interactions with both strands of the native β-sheet may be disrupted by the D178N mutation, thereby potentially affecting its stability. Spin-labeling ESR studies of D178N PrP indeed showed that the D178N mutation increases instability in S2 [139]. Crystal structures of D178N PrP with either M129 or V129 show little difference with the overall static

fold of WT PrP [75]. Differential packing of the monomers, however, indicates that the native β-sheet may combine to form an intermolecular four-stranded sheet with M129, but not with V129.

The experimental findings indicating that the D178N mutation affects PrP^C stability and promotes aggregation have prompted several MD studies studying the effect of the mutation on the PrP structure. To study the local structural effects, Billeter and Wüthrich performed short (0.5 ns) simulations of D178N, E200K mouse PrP combined with either M129 or V129 [140], in a small sphere of water. As expected, interactions with Y128 and R164 were disrupted (in contrast to the equivalent WT PrP simulations), but no further instability was found. Subsequent 1.5 ns explicit solvent MD simulations of WT mouse PrP (three trajectories) and D178N mouse PrP (two trajectories) also showed no differences in flexibility or conformation [141]. Interestingly, the R164-D178 salt bridge was only populated for 15% of the time in the most stable WT simulation in this study. The authors concluded that the R164-D178 salt bridge may not be important for WT PrP stability, but these simulations seem too short to substantiate such a statement.

Several MD studies were also run under conditions intended to perturb the starting structure further (in addition to the mutation). At high temperature, implicit solvent MD simulations of mouse PrP, HA was stable in WT but not in D178N PrP [142]. The authors attributed this to changes in the charge distribution that affect internal salt bridges in HA. In explicit solvent high temperature MD simulations of human D178N PrP (in combination with M129 or V129), however, no significant differences were observed [143]. In order to reveal weaknesses in the conformation of the globular domain, Barducci et al. performed simulations of WT and D178N mouse PrP in a hydrophobic environment (a solution of CCl_4) [144]. Multiple simulations of 3–8.1 ns revealed little difference in the overall conformation for WT PrP. With the D178N mutation, however, the S1/S2 β-sheet became unstable, presumably due to the lack of interactions with R164 and Y128. The weakening of the β-sheet in relation to disruption of the D178-R164 and D178-Y128 interactions was later confirmed using the metadynamics simulation technique [62].

4.2 Mutations in the Hydrophobic Core

The three-dimensional structures of PrP of a variety of species have indicated that a conserved core of hydrophobic residues may confer stability to the globular domain [145]. This hydrophobic core consists of comprehensive interactions between HB and HC, interactions between HC and the loop preceding HA as well as contacts between the native sheet and the rest of the globular domain (Fig. 1b). Four mutations in residues that are part of the hydrophobic core have been related to familial prion diseases in humans: V180I, F198S, V203I, and V210I [123, 146–149]. Further, Thr183 forms additional interactions with the hydrophobic core, and its mutation to Ala causes familial CJD [150]. Experiments on mouse

PrP mutants indicate that thermodynamic stability of PrP is significantly reduced for T183A and F198S and somewhat reduced for V180I [136]. For V180I, V210, and F198S PrP, it was found that a folding intermediate increased in the population relative to the native fold, further indicating that these mutations cause instability [89, 151]. Studies of V203I PrP are limited, but may suggest an effect on stability as well [152]. For T183A, the observed instability may be related to the loss of the hydrogen bond between Thr183 (in HB) and Y162 (in S2). The replacement of a hydrophobic residue with a hydrophilic residue in F198S PrP leaves a "gap" in the hydrophobic core between HB and HC. The other three mutations are more conservative (Val to Ile), and it is therefore more difficult to interpret their potential effects from the WT structure alone.

To investigate the influence of the disease-related mutations in the hydrophobic core on the conformation and dynamics of recPrP, Van der Kamp and Daggett recently performed extensive MD simulations [126]. In a comparison of three 50-ns runs for each mutant with equivalent simulations of WT human PrP (res. 90–230), all mutations were observed to have some effect on structure and stability. In line with the effect on the thermodynamic stability of PrP, T183A and F198S significantly increased the flexibility of the globular domain, including the parts of HB and HC that are particularly stable in WT PrP (Fig. 4a). For F198S, flexibility was most strongly affected in the loop between HB and HC and the adjacent parts of the helices, as could expected based on the structural role of the Phe198 side chain [145]. Interestingly, a further significant effect was a shift of the native β-sheet (~20 Å from the mutation site) away from the rest of the globular fold. Such as shift was also observed for T183A and, to a lesser extent, for V180I, and could be related to a loss of the hydrogen bond between Thr183 and Tyr162. The addition of an extra CH_3 group in the Val to Ile mutations can cause steric crowding in the hydrophobic core. For V180I and V210I, this appears to cause a change in the hydrophobic packing of residues between HB and HC, which in turn causes Phe198 to move out of the hydrophobic core during the simulations. Further crowding effects also cause a reduction of the hydrophobic contacts between the S1-HA loop and HC. In turn, this change can lead to HA moving out into solvent, similarly to what was observed in acidic conditions [59]. V203I was also observed to cause these effects, albeit to a lesser extent.

In one of the V180I simulations, a change in positioning of HA and the S1-HA loop is preceded by the addition of an extra strand onto the native sheet (Fig. 4b). This results in a similar early misfolded state as observed in simulation at acidic pH. In this conformation, significant parts of the original hydrophobic core are exposed to solvent, which will contribute to its aggregation propensity. Similar conformations related to early misfolding were also found in simulations of V210I. At the same time, the core of HB and HC in the V210I simulations showed similar stability and conformation as in the WT PrP simulations at three pH ranges [59]. These similarities between conformation and early misfolding are in line with the findings that PrP^{Sc} in patients carrying the V210I mutation consist of both V210I and WT PrP [153]. In contrast, the significant instability and conformational changes observed for T183A and F198S, also observed in coarse-grained MD simulation

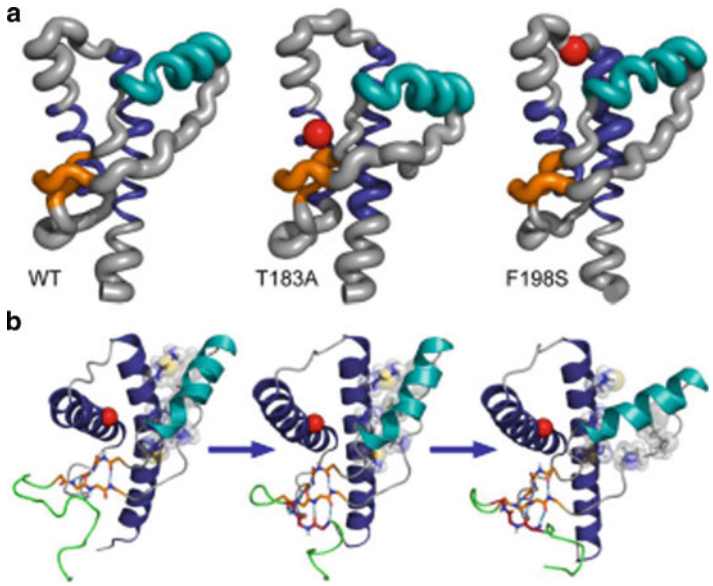

Fig. 4 Changes in conformation and flexibility caused by pathogenic mutations [126]. (**a**) Typical conformation and flexibility for WT PrP, T183A PrP and F198S PrP. Only the globular domain (res. 128–228) is shown. Flexibility is indicated by the *thickness* of the *ribbon* and the mutation site is indicated by a *red sphere*. (**b**) Early misfolding events in a simulation of V180I PrP. First, an additional strand appears on the native sheet. Thereafter, hydrophobic contacts between HC and the S1-HA loop are lost and HA moves out to solvent. Mutation site is indicated by a *sphere*. For clarity, res. 90–112 are omitted

of T183A [154], may indicate a different disease mechanism. Cell studies indicate that the instability of the globular fold interferes with GPI-anchor attachment and subsequent cellular processing [155, 156]. In vivo, these mutations typically cause a prolonged duration of disease [157] and can lead to abnormal PrP^{Sc} formation [158]. Although correlations between simulation of recPrP and disease are speculative, they indicate how MD simulation helps to uncover detailed molecular effects that may be important for the development of familial prion disease.

5 Other Applications

5.1 In Vivo Modifications

Although most MD simulation studies and many experimental studies have focused on recPrP, the protein can undergo important post-translational modifications in vivo. After translation and transport to the endoplasmic reticulum, N-glycosylation may occur on two different sites in the globular domain (Asn181 on HB and

Asn197 in the HB-HC loop), yielding un-, mono-, and diglycosylated forms of PrP [159]. Modification of the initially attached high-mannose glycans occurs in the Golgi and creates a very diverse set (> 400) of PrP glycoforms [160]. The effect of glycosylation on the structure of PrP was first studied by MD simulation [55]. Short (1.8 ns) simulations of a homology model of human recPrP (res. 90–230) with or without typical complex glycoforms indicated that glycosylation did not perturb the structure, and may even stabilize it somewhat. Although these simulations were too short to capture potential large conformational changes, NMR studies on bovine PrP purified from brain extracts indicated that the structure of glycosylated PrP (including the sugar portion of the GPI-anchor) is very similar to recPrP [161]. Recently, longer extensive MD simulations of diglycosylated PrP were reported [53]: DeMarco and Daggett performed 15-ns simulations of human PrP (res. 90–230) with complex glycans at neutral and low pH and concluded that the conformation and dynamics of PrP were not affected. It is possible, however, that contacts between the glycan attached to Asn197 and HA perturb pH-induced misfolding, which may affect binding efficiency to PrP^{Sc} and the morphology of the fibrillar aggregate [53]. Furthermore, the authors also reported equivalent simulations of diglycosylated PrP attached to a membrane via the GPI-anchor. This revealed that protein-membrane interactions probably only occur with the flexible N-terminus.

Another possible post-translational modification is the oxidation of methionine side chains, which can occur in vivo due to the presence of various reactive oxygen species. It is a reversible process due to the action of methionine sulfoxide reductases, but the methionine sulfoxide content of proteins increases with age [162]. In contrast to PrP^{C}, a large fraction of methionine side chains were found to be oxidized in PrP^{Sc}, especially at Met213 [163]. A more recent study, however, concluded that Met213 is found in its oxidized state equally in PrP^{C} and PrP^{Sc} [164]. It is therefore unclear whether methionine oxidation can be involved in triggering misfolding or stabilizing prion aggregates or not. To investigate if methionine oxidation could destabilize the PrP^{C} fold, MD simulations were performed of human PrP (res. 125–228) without Met oxidation, with Met213 oxidized and with Met206 oxidized [58]. Both residues are buried in the hydrophobic core of the native PrP structure. The simulations (two runs of 80 ns for each species) indicated that methionine oxidation did not have a significant effect on the local structure, but a shift to more flexible conformational states occurred. Seemingly, the effect was transmitted to more distant regions in the structure, such as the S2-HB and HB-HC loops. It was suggested that methionine oxidation could trigger misfolding. This hypothesis was later corroborated by experimental studies of recPrP that used norleucine and methoxinine as analogues of the hydrophobic, non-oxidized form or the hydrophilic oxidized form of methionine, respectively [165]. The norleucine variant exhibited a stabilized α-helical structure and low aggregation propensity, whereas the methoxinine variant largely consisted of β-structure and had a high tendency to aggregate. Recently, experiment and MD simulation were used to determine that the M206S and M213S mutations also destabilized the native PrP fold and enhanced aggregation propensity [166].

5.2 The Effect of Small Molecule Ligands

The interaction of PrP with small molecule ligands can be of great interest with respect to the development of possible drugs targeting prion diseases, for example by inhibiting misfolding. MD simulations can be used in order to explain and rationalize the effects of small molecules. One example of this is the effect of the "chemical chaperone" trimethylamide *N*-oxide (TMAO) on PrP misfolding. Experimentally, it was found that addition of TMAO efficiently reduced PrP^{Sc} formation in mouse neuroblastoma cells (75% reduction with 120 mM TMAO) [167]. Simulations in the presence of 1 M TMAO were performed with hamster recPrP (res. 109–219) [168], starting from either the native conformation or a previously determined pH-induced misfolded conformation [56]. Both simulations were carried out with protonation states of the amino acids corresponding to the strongly acidic pH regime, to perturb the PrP structure. TMAO itself was not protonated (whereas its pK_a is ~4.7), as this is likely to cause destabilization rather than stabilization of protein structure [169, 170]. Starting from the misfolded conformation, the extended sheet is disrupted and PrP regains contacts that had been lost during the pH-induced misfolding (Fig. 5). In the simulation starting from the native structure, no misfolding is observed. In both cases, the hydrophobic residues in the flexible N-terminus formed an Ω-loop that prevents this region from forming

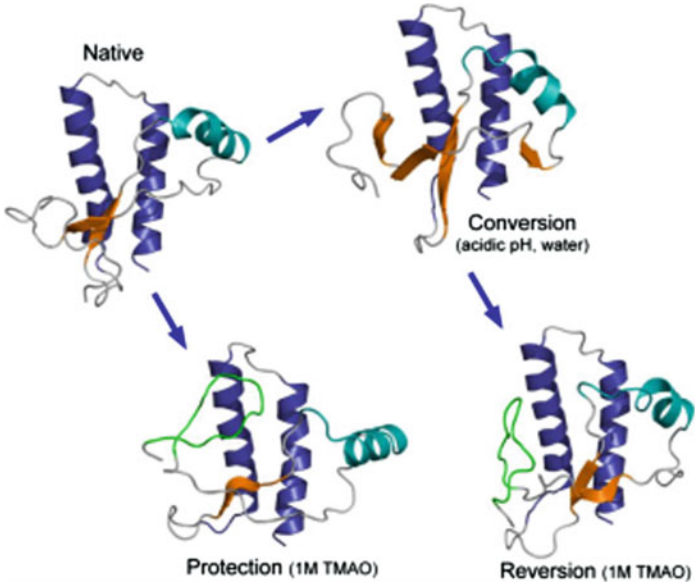

Fig. 5 The effect of TMAO on PrP structure and misfolding. Starting from natively folded hamster recPrP (res. 109–219), simulation at acidic pH in water causes conversion to a misfolded form [56]. In the presence of 1 M TMAO, however, the structure is protected from misfolding (protection) and the extended sheet in the misfolded conformation dissolves (reversion) [168]. An Ω-loop conformation (*green*) in the flexible N-terminus is observed in the presence of TMAO

additional β-strands. The simulations further confirmed that the stabilizing action of TMAO is likely to be indirect: interactions of water molecules with the peptide backbone become less favorable in the presence of TMAO [171, 172].

Whereas the osmolyte TMAO cannot be used in vivo due to the toxic effect of trimethylamine, other compounds that inhibit formation of PrP^{Sc} can be administered to mammals. An example is *N*,*N*′-(methylenedi-4,1-phenylene)bis[2-(1-pyrrolidinyl) acetamide], or GN8. This compound was found to inhibit PrP^{Sc} production in vitro and prion-infected mice treated with GN8 showed prolonged survival [173]. The molecule was selected based on virtual screening of compounds that would bind in the region of the HA-S2 loop and the HB-HC loop of mouse PrP. According to the virtual screening results, GN8 forms hydrogen bonds with Asn159 and Glu196, thereby cross-linking the two loop regions. Yamamoto and Kuwata [174] first performed a 100-ns MD simulation of the initially obtained binding mode in water to refine the PrP-GN8 structure further. The binding mode obtained agreed with NMR chemical shift data [173]. Also, the flexibility of the PrP structure was decreased in comparison with the equivalent simulation without GN8. To examine the stabilizing effect of GN8 further, simulations were also performed of mouse PrP in 6 M urea, either with or without GN8 bound [174]. These simulations indicate that the presence of urea destabilizes all three helices, whereas binding of GN8 prevents this destabilization to some extent. GN8 also appears to act as a "chemical chaperone" that reduces the flexibility of the native PrP structure and increases its stability.

6 Conclusions and Outlook

When properly prepared and executed, MD simulations are able to capture realistic information on the dynamics and conformational ensembles of PrP. The benefit of such simulations comes from the unique spatial and temporal resolution, providing significantly more detailed information than is available from experiment. Early MD simulations have already provided insight into local and global changes, including possible steps in the misfolding and aggregation of PrP. Advances in computer speed and algorithms have made it possible to perform more and longer simulations in recent years. This has opened up the possibility for increased sampling of the conformational dynamics of PrP. These new, extensive MD simulations have contributed significantly to our knowledge of the detailed atomic-level molecular mechanisms that are involved in the response to decreases in pH, single residue mutations, in vivo modifications, and small molecules.

The continuing increase in computer resources makes it possible to simulate bigger systems and longer timescales. This will allow, for example, for a more detailed characterization of the conformation and dynamics of PrP^{C} under near-physiological conditions: glycosylated and bound to a membrane. These studies are currently underway in our group. Further, direct simulation of the process which is at the very heart of prion infection, conversion of PrP^{C} on a template of PrP^{Sc}, is also now possible.

The detailed information, gained from MD simulations, on the early mechanism of misfolding and related instabilities can be exploited for the design of small molecules or peptides that may serve as novel diagnostic tools and drugs. Once such molecules are designed, MD simulations can be employed to test and explain their action in detail. Overall, we expect that MD simulation methods will continue to be a valuable tool to provide information on various different aspects of the PrP: the behavior and conformation of cellular PrP, misfolding of PrP, influence of mutations, and development of prion disease diagnostics and therapies.

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Chemical Biology of Prion Protein: Tools to Bridge the In Vitro/Vivo Interface

Ralf Seidel and Martin Engelhard

Abstract Research on prion protein (PrP) and pathogenic prion has been very intensive because of its importance as model system for neurodegenerative diseases. One important aspect of this research has been the application of chemical biology tools. In this review we describe new developments like native chemical ligation (NCL) and expressed protein ligation (EPL) for the synthesis and semisynthesis of proteins in general and PrP in particular. These techniques allow the synthesis of designed tailor made analogs which can be used in conjunction with modern biophysical methods like fluorescence spectroscopy, solid state Nuclear Magnetic Resonance (ssNMR), and Electron Paramagnetic Resonance (EPR). Another aspect of prion research is concerned with the interaction of PrP with small organic molecules and metals. The results are critically reviewed and put into perspective of their implication for PrP function.

Keywords Biophysical monitors · Chemical synthesis of proteins · Copper binding · Expressed protein ligation · Interaction of PrP with small molecules

Contents

R. Seidel and M. Engelhard (✉)
Max Planck Institut für Molekulare Physiologie, Otto-Hahn-Str. 11, 44227 Dortmund, Germany
e-mail: martin.engelhard@mpi-dortmund.mpg.de

Abbreviations

EPL	Expressed protein ligation
NCL	Native chemical ligation
PrP	Prion protein
PrP^{C}	The cellular isoform of prion protein
PrP^{Sc}	The pathogenic or scrapie isoform of prion protein
PTS	Protein *trans* splicing
rPrP	Recombinant prion protein
SPPS	Solid phase peptide synthesis
TEV	Tobacco etch virus
TSE	Transmissible spongiform encephalopathy

1 Introduction

The chemical manipulation of molecules of life in order to understand their biological function has been the subject of intensive research since almost the beginning. One of the first highlights of peptide synthesis is connected with the name of Emil Fischer, who has revolutionized at the beginning twentieth century the chemistry of peptides, nucleic acids, and sugars, and who coined the *Lock and Key* principle of enzyme action [1]. In further developments the synthesis of small peptides like oxytocin [2] and subsequently insulin [3, 4] was accomplished, which allowed one to elucidate their functional activity and to study the properties of analogs. The introduction of solid phase peptide synthesis by Bruce Merrifield further advanced the field [5, 6], as it provided an easy access to peptides and, importantly, to small one domain proteins. This breakthrough was certainly a decisive highlight of natural product chemistry; however it did not deliver larger proteins which could be applied to answering further biological questions. The situation did change when fragment ligation became possible which produced a native peptide bond [7, 8]. This so-called *native chemical ligation* possesses such inherent chemical properties that it is possible to combine it with recombinant expressed proteins (expressed protein ligation [9, 10]). With these methodological advancements the manipulation of proteins in a cellular context is now within reach, thereby opening another aspect of chemical biology. Both approaches, small molecule interference and (bio) synthetic methods, have been applied in the field of neurodegenerative diseases in general and prion diseases in particular.

Chemical biology methods have been utilized in three different aspects of prion protein (PrP^c) research. The method was used for the structure determination of PrP and the mechanism of PrP–prion (PrP^{sc}) conversion. Furthermore, the question of infectivity can only be answered unequivocally if PrP preparation are used which have been obtained either by heterologous expression or by chemical synthesis. A further line of experiments was directed to simulate PrP posttranslational modifications like attachment of a GPI-anchor or glycosylation. Finally, small medicinally compatible molecules have been used to interfere with the PrP–PrP^{sc} conformational switch. In this review we will address these chemical biology aspects of prion research.

2 Chemical Synthesis of Proteins

The chemical synthesis of proteins is presently a well established method which relies first on an orthogonal protecting group scheme. An orthogonal protection scheme has been defined by Barany and Merrifield: *each class of* (protecting) *groups can be removed in any order and in the presence of all other classes*) [11]. This principle has not only been applied in peptide chemistry but also found entry in chemistry and protein chemistry (e.g., introducing specific protease cleaving sites). Protection schemes commonly used are based on the fluorenylmethoxycarbonyl (Fmoc) or the butyloxycarbonyl (Boc) group (for a useful book on solid phase peptide synthesis see [12]). The Fmoc moiety can be removed from α-amino groups by mild bases, such as piperidine, but is stable under acidic conditions. Therefore the linker to the solid support (a polystyrene based resin) is, like the side chain protecting groups, acid labile. On the other hand, the Boc group is acid labile and can be removed by trifluoroacetic acid. Removal of side chain protecting groups and concomitant cleavage from the resin is achieved by hydrogen fluoride. Because of the hazardous nature of HF the Boc protection scheme is used only in a few laboratories. However, it should be noted that the Boc chemistry allows faster coupling steps (10 min vs 60 min for Fmoc chemistry) and leads to higher purities for larger peptides. After the first amino acid has been attached to the solid support via its carboxyl group in repetitive reaction cycles the side chain protected peptide is assembled. The reaction cycle consists of (1) deprotection of the α-amino group, (2) activation of the next amino acids via, e.g., active esters, and (3) coupling of this amino acid to the α-amino group. These cycles are repeated until the peptide is assembled. At this stage the peptide is cleaved from the resin and subsequently purified by, e.g., HPLC. Since the purification occurs only after the peptide has been assembled the coupling yields have to be quite high. They are usually around 99.5%. Peptides of 60–70 amino acids are normally accessible. Overall yields of even larger peptides become quite low and also the purity of the final product may not be satisfactory. It is therefore advisable to switch to fragment coupling schemes if the target protein exceeds this size.

2.1 Native Chemical Ligation

Peptide fragment coupling has been in the focus of peptide chemists for a considerable time. However, first attempts were not very successful because of the protected peptide fragments which turned out to be quite insoluble in most solvents. Also, for fragment coupling, using a solid phase matrix turned out not to be practicable because of very poor (if any) yields. The situation changed when new methods were developed which could be performed with unprotected peptides or proteins. The most widely used method, native chemical ligation, relies on thioester coupling. The chemistry was partly elucidated by T. Wieland who in the early 1950s analyzed the intra-molecular displacement of S-acyl groups in peptides [13]. Forty years later, using this kind of chemistry, S. B. Kent developed a method which allowed coupling of unprotected fragments under aqueous conditions [7] (for a recent review see [8]). This new innovative reaction scheme resolved the problem of solubility while generating a native peptide bond (see Fig. 1).

In order to perform a native chemical ligation, two prerequisites have to be fulfilled. First, the C-terminal fragment (**2**) has to contain a Cys residue (which in principle could also be Ser, although it is less reactive) at the N-terminus. The N-terminal fragment (**1**) possesses a C-terminal thioester. The reactivity of this activated carboxyl group can be tuned by proper selection of the thiol substituent. For example, aromatic groups are more reactive than alkyl groups. The mechanism of peptide bond formation encompasses two steps. In a first reversible reaction the

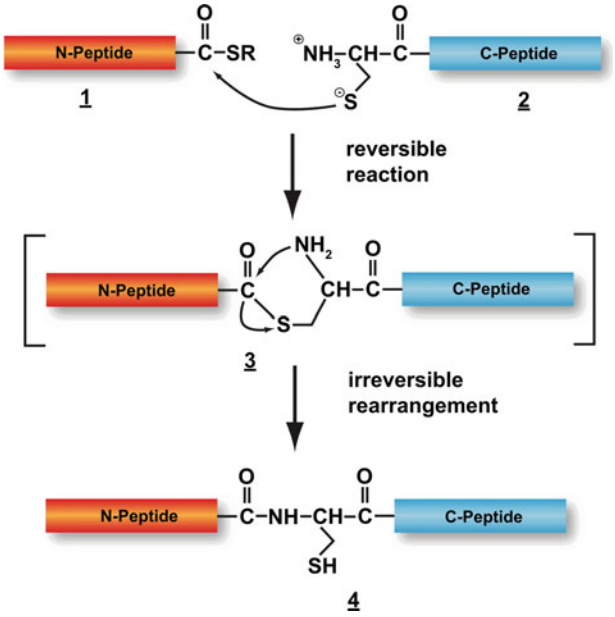

Fig. 1 Scheme of native chemical ligation (NCL)

Cys thiol attacks the carboxyl thioester, forming a new reactive thioester linking the two fragments by the Cys side chain (**3**). In an S → N acyl shift via a five membered ring, like that in the Wieland reaction [13], the free amino group of Cys replaces the thioester bond to form in an irreversible reaction an amide bond (**4**). These two reactions, first reversible subsequently irreversible in nature, enables fragments to couple even if other Cys residues are present in one or both of the two fragments [14]. This selectivity is due to the fact that main chain Cys cannot undergo an S → N shift because of its lacking primary amine. Instead, free thiols (e.g., mercaptoethanol) which are added to the reaction mixture restore the two educts.

Using an orthogonal protection scheme the method can also be applied to multiple fragment coupling. Several examples exist in the literature in which the method has been applied. For example, the small G-protein Ras, consisting of 166 amino acids has been successfully synthesized from three fragments [15]. From the work of the Kent laboratory the synthesis of lysozyme and its high resolution structure determination shows that chemical synthesis of proteins yield purities similar to those which have been recombinantly prepared [16]. In another interesting example, Kent and coworkers synthesized both D- and L-kaliotoxin (an inhibitor of the Kv1.3 voltage-gated potassium channel). The crystals of the racemic mixture diffracted to 0.95 Å which enabled structure determination by direct methods [17].

Principally two strategies to couple the fragments can be followed. In a sequential scheme one starts with the C-terminal peptide which is coupled to the next fragment and so on until the whole protein is assembled. This design requires the N-terminal protection of the incoming peptides. In the convergent synthesis proposed by Bang et al. “the two halves of the target sequence are prepared from multiple peptide segments and condensed in a final step to give the full-length polypeptide chain” [18]. Using this latter approach the authors described the synthesis of crambin from six fragments using kinetically controlled ligations (18).

These examples illustrate that the chemistry of thioesters can be successfully exploited for the synthesis of proteins. Also, in nature thioesters are very often used; e.g., in catalysis or in reactions for post translational modifications like e.g., ubiquitination or the attachment of a glycosylphosphatidylinositol (GPI) anchor. The prion protein is a prominent example for the latter modification. PrP possesses two signal sequences, one of which is responsible for the ER import and the other, a hydrophobic transmembrane helix, for the attachment of the GPI anchor (see [19, 20] and literature cited therein for recent reviews on the biosynthesis of PrP). The preformed GPI-anchor is attached to the target protein at the luminal membrane of the ER. The reaction is catalyzed by GPI-transamidases in a two step mechanism. In the first step the GPI signal sequence is cleaved by the attack of a Cys residue to generate an activated thioester resembling mechanistically the first step of native chemical ligation (Fig. 1). This intermediate is attacked in a nucleophilic reaction by terminal ethanol amine moiety of GPI to yield the mature GPI-anchored protein which subsequently is transferred via the Golgi network and vesicular transport to the outer leaflet of the cellular plasma membrane.

2.2 Expressed Protein Ligation

Native chemical ligation experienced a decisive further development when it was combined with intein technology. Inteins are identified in all three domains of life and could be found in the family of homing endonucleases (for a review see [21, 22]). The name intein is derived from intron/exon organization of DNA where the intron is spliced before RNA maturation. In the case of inteins the protein is expressed in full length consisting of protein intron (intein) and two flanking peptides (exteins). In a posttranslational reaction the intervening intein is cleaved out and the flanking N- and C-terminal peptides (N-extein and C-extein, respectively) are fused together to form a natural peptide bond (reviewed in [23]). A general feature of the intein domain is its two lobe structure which brings the N- and C-termini in close proximity. Important catalytic residues are an N-terminal Cys, a C-terminal Asn, and another Cys at the N-terminus of the C-extein (Fig. 2). In the catalytic core is a conserved aspartate which is crucial for the synchronized multistep enzymatic reaction [24]. This multistep reaction sequence starts with an N → S acyl shift (in some inteins this Cys is replaced by Ser) transferring the N-extein to the Cys side chain. In the second step this thioester is attacked in a transesterification reaction by another Cys (or Ser) residue which resides on the N-terminus of the C-extein. In this β-branched intermediate Asn cyclizes to C-terminal succinimide, thereby liberating the central intein. In the last reaction an S → N acyl shift occurs, generating the mature protein. Steps 1, 2, and 3 are in principle reversible whereas the cleavage reaction (step 3) is irreversible. The overall reaction has been termed cis-splicing to differentiate it from trans-splicing which uses so-called split inteins. Here, the intein domain is split into two halves which can bind together with high affinity. An example is DNA polymerase III from *Synechocystis sp*. PCC6803 which is only activated after the trans-splicing

Fig. 2 Structural model of *Mycobacterium tuberculosis* recA mini intein (PDB 2IMZ) after the splicing reaction. The N-terminal Cys is in close proximity of the C-terminal succinimide

reaction [25]. The occurrence of split inteins has been exploited for conditional protein splicing reactions which can, e.g., be performed on the cellular level (see below). It should be noted that intein type splicing is also found in other protein families. An important example concerns autoprocessing of hedgehog proteins where a splicing reaction occurs. But instead of fusing with C-extein, a cholesterol lipid is attached [26]. The above examples show that nature uses intein type reactions fairly diversely. It is also evident that this kind of sulfur chemistry is quite similar to that of native chemical ligation. Consequently, T. Muir and coworkers developed a method which they termed expressed protein ligation (EPL). EPL enables the combination of recombinant proteins (or chemically synthesized peptides) with proteins (or peptides) from other sources. In the following the mechanism of expressed protein ligation is discussed in more detail (Fig. 3).

As exemplified above, a ligation needs an N-terminal Cys and a C-terminal thioester as prerequisites. In chemical synthesis these two conditions are obviously easily achieved. However, recombinant proteins are synthesized with an N-terminal Met and the C-terminus is just a free carboxyl group. There are various methods available to generate an N-terminal Cys. In a commonly used strategy a specific protease cleaving site is introduced. The Tobacco Etch Virus (TEV) protease recognizes the sequence Glu-Asn-Leu-Tyr-Phe-Gln-(Gly/Ser) and cleaves the

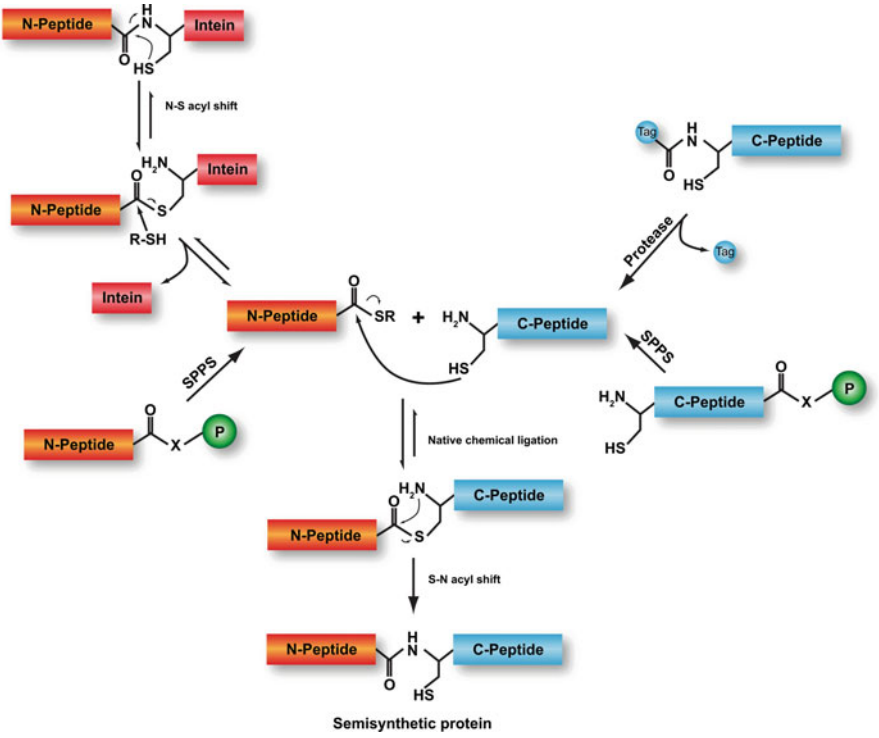

Fig. 3 Scheme of expressed protein ligation

C-terminus of Gln. The next amino acid, which generally is Gly or Ser, can also be replaced by Cys; thus a TEV cleavage generates a free N-terminal Cys. An advantage of this approach is the possibility to place an affinity tag (e.g., Strep-tag, Histidine-tag, FLAG-tag, or GST-tag) in front of the cleavage sequence, thereby facilitating the purification. The active thioester can be obtained by the intein technology.

A general strategy is depicted in Fig. 3. The chemistry shown in the central part of this figure corresponds to that already described in Fig. 1. Important are the two branches for accessing the precursors. The N-terminal peptides can either be prepared by recombinant technology as described above or by chemical synthesis using solid phase peptide synthesis. If proteins are expressed in a host organism like *E. coli*, yeast or insect cells, an intein fusion construct has to be designed. Commercially available intein constructs carry a mutation at the Asn (normally Ala) residue and/or Cys. These mutations prevent the intein reaction cascade proceeding to the end because the cleavage reaction (succinimide formation) cannot occur. However, in the presence of thiol reagents the β-branched thioester is cleaved and a new thioester is formed. This product can be used for subsequent native chemical ligation. To facilitate the purification of the final ligated product the C-extein often comprises an additional purification tag like a chitin binding domain and/or a His tag. With this strategy it is possible to bind, e.g., the precursor intein construct on a chitin matrix and subsequently cleave the protein of interest with appropriate thiols from the matrix, while forming the thioester. In principle, it is also possible to perform the ligation on the resin which would directly liberate the final product from the column.

The discovery of split inteins further advanced the scope of EPL into the realm of conditional protein splicing. As outlined above, certain inteins can be split into two halves. An advantage for a general applicability concerns the observation that the split site is asymmetric. This opens an access to chemical synthesis of the smaller intein half and hence an easy route to C-terminal modification of proteins with, e.g., fluorophores, lipid anchor, or other posttranslational modifications of proteins.

An intriguing new development relates to conditional protein splicing [27]. In this scenario the property of the immunosuppressant rapamycin is utilized to bind simultaneously to two different proteins (FKBP and FRB) with high affinity. If FKBP and FRB are linked to the N- and C-termini of the intein–extein halves (see Fig. 4 for the protein constructs) the addition of rapamycin would trigger the formation of a ternary complex, thereby bringing the intein halves into close proximity. In a consecutive reaction the two exteins can combine to generate an active or, e.g., fluorescent labeled protein. Since both halves can be expressed in mammalian and other cells, cellular functions can be studied.

Another example of conditional protein splicing using light as trigger reagent has been published by Pelois and Muir [28]. Based on a photocleavable group, the authors were able to obtain spacial and temporal control on protein function. Although this method is still in its infancy, it promises to become quite important as light as trigger is invasion free and has the additional advantage of being almost

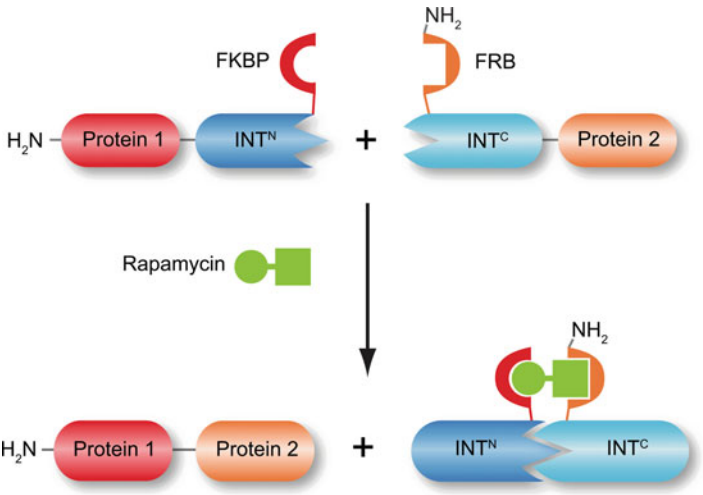

Fig. 4 Scheme of conditional protein splicing

instantaneous. It is also foreseeable that these kinds of approaches could also be applied to a whole organism as has been shown with channel rhodopsins in optogenetics (reviewed in [29]).

3 Chemical Synthesis of PrP

Before we discuss approaches for the synthesis of the PrP the overall structure should be explained. Mature PrP, a protein of about 240 amino acids, exhibits three domains (see [30] for primary sequence and posttranslational modifications of hamster PrP). The N-terminal domain is an intrinsically unstructured fragment with around 100 amino acids. It harbors five octarepeats whose function is unknown. The central core of PrP is structured with mainly α-helical elements. It is followed by the C-terminal fragment which is equipped with the GPI anchor. Further posttranslational modifications are two carbohydrate moieties which are connected to Asn residues at the second helix (α2) of the structured domain. Because infectivity has been associated with the central core (for a review see [31]), most chemical work has been focused on this region.

3.1 Chemical Synthesis of the PrP Peptide Core

The chemical synthesis of partial fragments of PrP was first undertaken by Prusiner and colleagues [32]. In 1995 Zhang et al. [33] describe the partial synthesis of

Syrian hamster PrP using Fmoc chemistry. The longest peptide ranged from residue 90 to 145. Smaller fragments from this region were also synthesized. The peptides were analyzed using various methods like NMR- or CD-spectroscopy. The authors could show that, in suitable aqueous buffers with organic solvent additives, α-helical structures were obtained. Under more physiological conditions in the presence of NaCl, β-sheet structures were stable, which had a tendency to aggregate into protease resistant fibrils. In a subsequent publication a 56mer peptide (Syrian hamster 90–145) was chemically synthesized and its influence on the aggregation behavior of PrP analyzed [32]. The authors conclude that the peptide when mixed with PrP^{C} induces PrP^{Sc} properties like forming fibrous aggregates. Interestingly, the peptide in a β-sheet rich conformation did not have an effect on PrP^{C} conformation, whereas a sample with random coil structure did. The results are in line with the notion that the first two helices α1 and α2 are implicated in α-helix → β-sheet conversion.

Another peptide from Mouse PrP comprising residues 89–143 (MoPrP89-143 which corresponds to human HuPrP(90–144)) was mutated in position P101 to Leu [34]. A Japanese patient carrying an amber mutation in the corresponding codon 145 was reported to display a prion disease. When Kaneko et al. injected the peptide intracerebrally into transgenic mice, only those who received the β-form of the peptide developed the disease at an earlier onset. Comparing the results from the two papers on properties of the chemically synthesized peptides, they are somehow contradicting. Whereas in in vitro experiments only the random coil structure induced PrP^{C} prion like conformational changes, in in vivo experiments the β-structured peptides accelerated the initiation of the disease.

Continuing this work on the chemical synthesis of prion protein derived peptides, Prusiner and his coworkers also described the synthesis of various peptides encompassing residues 89–230 [35]. The peptides were synthesized by SPPS and NCL in good yields and purity. These peptides were selected because of the observation that a "mini prion" (PrP106) with deletions between 23–88 and 141–176 still retained in mice the ability to generate PrP^{Sc}. For the synthesis the two peptides were synthesized separately, one with a thioester, the other with an N-terminal Cys. After purification NCL led to the final product. After refolding the secondary structure were determined by CD-spectroscopy and Fourier Transform Infra Red Spectroscopy (FTIR) and proteinase K resistance was analyzed. It turned out that the chemically synthesized peptide, like the recombinant prepared PrP analog, displayed similar properties in its propensity to form β-sheet structures. In further experiments the authors introduced GPI-mimics at a C-terminal Lys. The strategy used an orthogonal protection scheme based on Boc chemistry. The C-terminal Lys was protected with the Fmoc group which can be removed by mild bases before cleaving the peptide from the resin. The free amino group was then modified either with biotin or with myristic acid. These two groups were chosen because biotin provided an independent epitope which would allow it to be distinguished from endogenous PrP^{C}. The lipid-derivatized proteins might be useful for in vivo studies on infectivity. In experiments with PrP-null neuroblastoma (N2a) cells the authors could show that the myristylated derivative was able to bind to the cell surface.

3.2 Approaches for the Synthesis of GPI Anchored PrP

The last example shows that chemical synthesis of proteins can be a useful tool to study pertinent questions in biochemistry. Its main advantage so far relates to its capability to introduce unnatural amino acids, specific isotope labels, biophysical monitors, or very important posttranslational modifications. The latter modification the introduction of a GPI anchor or a GPI anchor mimic onto PrP, has been the subject of further research. These experiments were not solely directed towards specific questions of PrP research but also towards development of a general method to modify proteins with a GPI anchor.

In Ball et al. [35] the GPI mimic was attached to the side chain of a Lys residue. The native GPI anchor is bound to the C-terminus via an amide bond to ethanolamine. This native linkage can be introduced by thioester chemistry which was exploited in two other laboratories.

Olschewski et al. [36] uses a semisynthetic strategy. Murine PrP(90–232) was expressed in *E. coli* as an intein fusion construct which also carried a His tag and chitin binding domain for purification purposes. The C-terminal peptide was synthesized by Fmoc chemistry and carried two palmitoyl fatty acids via an amide linkage to Lys residues. An additional TEV protease cleavable polyethyleneglycol polyamide building block was attached to the C-terminus of the peptide as solubilization tag. The denatured protein was refolded in a buffer containing L-arginine and glutathione disulfide (GSSG) for proper disulfide formation. The folding protocol was based on earlier published work on the NMR structure of unlipidated rPrP [37]. The CD spectrum was identical to that of rPrP and showed the expected α-helical content. Dilipidated rPrP bound readily to DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) vesicles and was taken up into the plasma membrane of murine neuronal (N2a) and human embryonic kidney (HEK293T) cells. Interestingly, the membrane attached rPrP is less susceptible for the $\alpha \rightarrow \beta$ transition. The project for the semisynthesis of rPrP with a GPI anchor mimic will enable experiments on the cellular level; specifically PrP uptake and turnover as well as the $PrP^{C} \rightarrow PrP^{Sc}$ conversion will become feasible.

A step further to the synthesis of native PrP was accomplished with the introduction of a GPI anchor module which can be attached to any recombinantly generated protein carrying a thioester [38]. The corresponding coding sequence consisted of yeast Kre5p as an ER import signal and the genes for human PrP (hmPrP) and GPI anchor signal sequence Gas1p (a GPI glycoprotein). Additionally, the coding sequence for a TEV cleavage site was introduced between a His-tag and a Flag-tag. The design of the PrP yeast expression system was based on previous work in which the post-translational import of PrP into the ER had been analyzed [39]. In another publication, in which copper utilization of yeast cells in dependence of PrP expression was studied, Li et al. described the expression of PrP on the cell surface [40]. However, no isolation and purification was performed. Schumacher et al. expressed the PrP-GPI fusion protein in yeast and could show that it is localized in the outer plasma membrane [38]. PrP-GPI could be purified

to homogeneity. In a subsequent step the fusion protein was cleaved by TEV protease producing a GPI synthon with an N-terminal Cys. A mass spectrometric analysis provided information about the core structure of the GPI anchor. It consisted of five mannose units, one inositol with an attached diacylglyceride and an additional ethanolamine phosphate. This method for the generation of a GPI module is generally applicable and might allow the elucidation of the family of GPI anchored proteins. It should be noted that the construct contains a free Cys residue at the ligation site which is in principle a target for introducing biophysical probes like fluorophores or spin labels expanding the scope of applications. This GPI-synthon was used to modify – in a trans-splicing reaction – the plasma membrane of living cells with green fluorescent protein [41].

Another route was chosen by Becker and coworkers ([42], reviewed in [43]) who chemically synthesized the glycosylphosphatidylinositol core. Chemistry of carbohydrates is quite challenging but recent developments enabled the synthesis of complex carbohydrates and glycoproteins (reviewed in [44]). The strategy used by Becker et al. to generate the GPI mimic involved the solution chemistry of the carbohydrate with a Cys coupled via its C-terminus to an ethanolamine of the first mannose. On the other side of the carbohydrate chain a stearoyl phosphate was attached to inositol. This proof of principle might lead to new approaches to elucidate PrP function and pathogenicity, although one has to keep in mind that the chemistry involved is highly challenging and up to now only possible in suitably equipped laboratories.

3.3 Introduction of Biophysical Monitors

The elucidation of PrP structure and function often necessitates the incorporation of biophysical probes. These monitors can be fluorescent dyes which enable one to study protein function both in vivo and in vitro by modern fluorescence methods. The incorporation of nuclear magnetic resonance (NMR) sensitive isotopes or spin labels opens the route to NMR and electron paramagnetic resonance (EPR) methodology. In the following these three methods will be briefly discussed in respect of their application in prion research.

3.3.1 Fluorescence Methods

There have been quite a few papers published which deal with fluorescence methods in prion research. Generally, three different approaches have been utilized. First, PrP chimeras with fluorescent proteins (e.g., green fluorescent protein, GFP) have been produced to study various aspects of cellular questions or protein folding. For example, fluorescence imaging techniques have been applied to study prion propagation in yeast (reviewed in [45]). Other examples concern attempts to study protein folding and the nature of aggregates. Kawei-Noma et al. analyzed the

dynamics of yeast prion aggregates in living cells by using fluorescence correlation spectroscopy (FCS) ([46], originally introduced into prion research in [47]). In more recent work the same laboratory provided evidence of fibrillar structures of Sup35 prions in yeast cells [48]. In this latter work the authors not only used fluorescence microscopy but also rapid freeze electron microscopy. The folding problem was further tackled by a couple of groups [49–52]. Using enhanced green fluorescent protein (EGFP)-tagged PrP in vitro oligomer formation was studied [49]. Fluorescence correlation spectroscopy (FCS) indicated that oligomers can already be obtained at relatively low sodium dodecyl sulfate (SDS) concentrations. These investigations are interesting, as they provide additional information about the mechanism of oligomers formation. However, one has to consider the limitations of this approach. Since the relatively large fluorescent protein is only attached either C- or N-terminally, immediate folding events cannot be observed.

This folding process can be studied if the fluorescent chromophore is directly incorporated into PrP. The fluorophore can be either an organic dye or tryptophan, the latter disturbing only minimally the native structure. Scheibel et al. mutated in the prion-determining region of Sup35p two positions (T158C and E167C) into Cys residues and modified them with fluorescent probes [53]. The fluorescent changes coincided with aggregate formation which also included the lag-phase. The initial steps of protein folding were investigated by stop-flow experiments [54]. Utilizing the Trp fluorescence of murine PrP(121–231)-F175W the authors observed extremely rapid direct folding of the C-terminus without folding intermediates. Multiple sites for fluorescence labeling with acrylodan was chosen by another group [55]. Denaturing experiments of full length PrP amyloids revealed that positions 127, 144, 196, and 230 exhibited cooperative unfolding whereas position 98 showed a different cooperative behavior. The authors conclude that at least two independent cooperative folding domains exist within the amyloid structure of PrP.

In a third approach, Lindquist and her colleagues used fluorescent imaging and optical trapping techniques to analyze the forces which determine the integrity of yeast prion fibrils [56]. The results revealed strong non-covalent interactions that preserve the fibril structure even if individual fibrils unfold.

3.3.2 Nuclear Magnetic Resonance

There are two different methods to analyze proteins by NMR spectroscopy. Most commonly used are techniques which afford a soluble protein whose size generally does not exceed one or two domains (about 25 kDa, for further reading refer to the Nobel lectures of R.R. Ernst [57] and K. Wüthrich [58]). With the development of larger magnets (1,000 MHz, 23,5 T) higher molecular weight proteins also become accessible for NMR experiments in solution. This size limitation is due to the molecular tumbling which averages out the chemical shift anisotropy at lower molecular weights. Increasing molecular weights decreases the spectral resolution which makes it more and more difficult to assign all amino acids. Obviously, membrane proteins, fibrils and aggregates are also not amenable to solution NMR

techniques. New advancements in NMR methodology enabled the latter classes of proteins to be studied by solid state NMR (ssNMR). In this case the chemical shift anisotropy can be canceled by spinning the sample at high speeds (15,000 Hz) around the magic angle (reviewed in [59]). Solid state NMR has been successfully applied to structural investigations in the prion field (see below).

NMR experiments afford isotope labeling with ^{13}C and/or ^{15}N which is normally achieved by expressing proteins in *E. coli* using minimal media (e.g., including ^{13}C-glucose, ^{15}N-NH_4Cl). Using ligation chemistry (see above) it also becomes possible to label only fragments within a protein, an advantage which reduces the problem of assignment.

It has been mentioned above that the first structure of the mouse prion core domain was determined by NMR in the Wüthrich and Glockshuber laboratories [37]. Subsequently, Wüthrich determined PrP structures from various sources including, e.g., cats, elk, chicken, or fugu [60–65]. The quintessence of these investigations was that global architecture is preserved. While comparing the NMR structures of horse PrP with that of other mammalian PrPs, the Wüthrich laboratory reported an interesting finding concerning differences in the loop connecting the β2 strand with the α2 helix [64, 65]. The authors argue that the well ordered loop of horse PrP might be a reason why there are no reports of TSE in horses. A similar conclusion was drawn from NMR experiments of human PrP Q212P mutant [66].

Recently, a comprehensive review on amyloid structures using ssNMR has been published by R. Tycko including the most recent results on PrP [67]. Measuring amyloids using ssNMR one has to keep in mind that the fibrils are generally polymorphic, giving rise to line broadening or multiple resonances for identical sites. This fact makes it difficult to determine the structure, but, e.g., information about dynamics or accessibilities can be extracted. Structural models might be feasible if electron microscopy data are taken into account. Most data were obtained from mammalian PrP, yeast PrP, and HET-s from *Podospora anserine* whose aggregation seems to have functional importance.

EPR- and hydrogen/deuterium exchange data indicated that the core of PrP fibrils encompasses residues about 164–220 and contains in-register parallel β-sheets [68, 69]. Studies using a uniformly labeled truncation mutant (PrP (23–144)) produced well-resolved signals in 2D-ssNMR [70, 71]. The immobilized core contained residues 112–141. Interestingly, the N-terminus displayed chemical shift values characteristic for random coils [70] (see below). Also, yeast prions were shown to contain in-register parallel β-sheet structures (see, e.g., [72] and literature cited therein) which are consistent with polar zippers between Gln and Asn side chains [73]. As mentioned above, HET-s prion form displays a functional role. Consequently, the ssNMR spectra revealed remarkably sharp signals which allowed the complete resonance assignment and a proposal for the structure consisting of β-helix motifs as found in certain monomeric proteins [74]. For a recent review see [75]. From these brief summaries it is obvious that NMR and ssNMR techniques can provide valuable information about the structure and dynamics of amyloids.

With methods like EPR, EM, or vibrational spectroscopy detailed structural information can be obtained, even from samples which cannot be crystallized.

3.3.3 Electron Paramagnetic Resonance

EPR-spectroscopy relies, like NMR spectroscopy, on magnetic resonance, in this case that of unpaired electrons. The method has been introduced into biochemistry by W. Hubbell [76, 77] and has since then successfully been applied in various fields of biochemical research (for reviews see [78, 79]). Generally, proteins, nucleic acids, or other biochemical compounds do not carry unpaired electrons. Therefore, this spin label has to be introduced into the target by chemical means. The organic nitroxide has been implemented as a suitable reagent. In order to introduce spin labels in a site directed manner, the sulfhydryl group of Cys is most commonly used to modify it with the methanethiosulfonate spin label (MTSSL) (see Fig. 4). Obviously, other naturally occurring Cys residues have to be removed by appropriate mutations. A limitation of the method so far is that it is only applicable in vitro because the reducing environment inside cells would immediately reduce the nitroxide to hydroxylamine. In EPR experiments various parameters can be determined. From the line width of the EPR signal one can deduce the mobility of the spin label which also reflects backbone dynamics. Addition of paramagnetic quenchers provides information about solvent accessibilities. Additionally the polarity of the immediate environment of the nitroxide label can be obtained. The method also yields intra- or intermolecular distances between two nitroxides or a single nitroxide and another paramagnetic center in the system. This latter property is quite important for the elucidation of amyloids. Distances can be determined from about 0.1 nm up to 8 nm if modern pulse techniques are applied.

EPR spectroscopy has been applied in prion research quite early (see [80] for a comprehensive review). However, only a few data are available from site directed spin labeling. In an early paper the aggregation kinetics of a short fragment (simian hamster PrP(113–120)) was studied [81]. The authors conclude on the presence of monomer peptide, amyloid, and amorphous aggregate and that amorphous material must be present in order to obtain amyloids. As mentioned above, Cobb et al. used EPR spectroscopy to analyze the molecular architecture of PrP [68]. The analysis of amyloids, fibrils, or aggregates by EPR spectroscopy poses some difficulties concerning distance measurements. Although normally only one spin label is introduced, in a fibril or amyloid spin labels from different molecules can be located in the vicinity of each other. Consequently, distance measurements could result in distribution of different distances interfering with reasonable conclusions. To overcome this constraint one could dilute spin-labeled molecules with unlabeled samples.

Most information from EPR experiments was gained elucidating the metal binding domain of PrP. It has been shown that Cu^{2+} can bind to the N-terminal fragment with various affinities. Cu^{2+} possesses a spin of 1/2, making it a suitable

EPR monitor for metal/protein interactions. Research on Cu^{2+} binding to PrP has been quite intensive (see below) which also included EPR experiments (see [80]). Most of these experiments were done on recombinant PrP or peptides derived from the N-terminus, especially from the octarepeat region. For further discussion on PrP metal binding site see next paragraph.

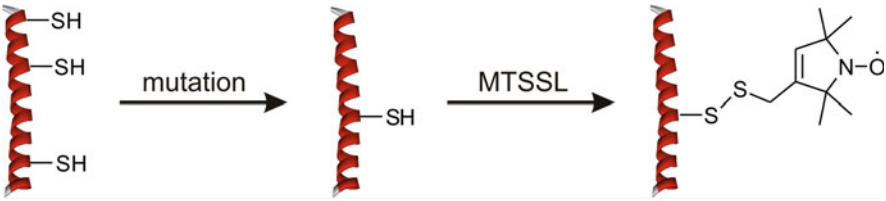

Fig. 5 Scheme of site directed spin labeling (MTSSL: Methanethiosulfonate Spin Label)

4 Interaction of PrP with Metals, Peptides, and Small Molecules

PrP displays triple domain architecture. The C-terminal domain harbors the GPI anchor and the central core undergoes the well known α-helix to β-sheet transition, whereas the N-terminal domain is intrinsically unstructured. The concept of intrinsically unstructured proteins or protein domains has been first developed by A. K. Dunker who also provided the means to identify these motifs by bioinformatic calculations (the concept of unfoldomics has been extensively reviewed by A. K. Dunker in various publications [82–84]). It turned out that proteins with intrinsically unfolded domains are involved in a variety of cellular processes, including, e.g., signaling, regulation, and cell cycle control. An intriguing property of this structural element is its plasticity conveying the ability to interact with a broad range of ligands. It seems that the folding process normally related to one protein chain after ribosomal translation is only triggered by appropriate ligands. Consequently, most intrinsically disordered proteins are found in the subclass involved in protein/protein binding [83]. The N-terminus of PrP is a typical case example. It can accommodate copper but, as most recent results show, it can also bind to β-sheet forming peptides. In the following these two aspects of PrP interaction with other molecules will be discussed briefly. A third class of binders, small organic molecules, probably interferes with the folding of PrP, and its binding motif should therefore reside in the central core.

4.1 Interaction of PrP with Metals

There is a vast literature about the interaction of Cu^{2+} and other divalent metals like Mn^{2+} or Zn^{2+} with the N-terminus of PrP (reviewed in [85, 86]). It has been

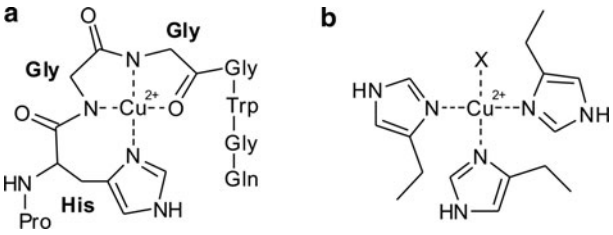

Fig. 6 Proposed PrP copper binding sites. (**a**) Component at low copper concentrations. (**b**) Component at high copper concentrations. The figure was adapted from [89]

recognized from early on that the N-terminus with its octarepeat region could harbor copper or other metal binding sites. These repeats contain His and Trp residues known as potential ligands of transition metals. First, experiments using chemically synthesized peptides from the octa and hexa repeat region of mammalian and avian PrP, respectively [87, 88]. These authors could already demonstrate that Cu^{2+} (with a dissociation constant (K_d) of about 6 μM) is preferentially bound and that one copper binds per repeat unit. Later work produced a more confused picture of metal binding. The dissociation constants varied substantially, depending on the method applied, the experimental conditions (e.g., pH, Cu^{2+} concentration, or additives like Gly), or the nature of samples (e.g., full length PrP or fragments, thereof). Measured K_d values fall in the range from micromolar to femtomolar [85], indicating that something is missing. Because this field, despite numerous publications, is still so controversial, we will discuss here only three aspects of copper binding: (1) possible Cu^{2+} binding motifs, (2) physiological copper concentrations, and (3) discussed physiological role of copper.

1. It seems clear that each octarepeat unit can individually bind one copper ion. The complete octarepeat can hold four copper atoms. From spectroscopic evidence it has been proposed that the ligands involved in this type of binding are His and Gly residues (see Fig. 6a). Cu^{2+} is coordinated to deprotonated nitrogen of the His side chain and those of the Gly peptide bond. The latter ligands afford a prior deprotonation which should be pH dependent. At low concentrations Cu^{2+} can choose an alternate binding site with His residues from different octarepeats [89]. Other workers locate this site proximal to the octarepeat [90, 91]. An additional binding component has also been discussed (e.g., [89]) (Fig. 6).
2. Physiological free copper concentrations are certainly decisive for determining a role for copper in PrP cellular function. It is important to recall the properties of Cu^{2+} in buffer solution. The solubility product (K_{sp}) of $Cu(OH)_2$ at physiological pH is in the order of 10^{-20}, i.e., rather small. It is therefore likely that it is bound to other compounds like, e.g., human serum albumin which binds copper in the picomolar range [92]. To evaluate whether PrP is a functional copper binding protein one has to determine the relative concentration of membrane attached PrP^C and that of albumin in the cerebrospinal fluid which is about 3 μM [93]. However, the free concentration of copper has not been determined. Inside cells

the copper concentration should be minimal (about 1 Cu atom/cell) because of the high binding affinities of copper proteins like superoxide dismutase.

3. Various functional roles have been attributed to copper in PrP physiology (briefly summarized also in [85]). It has been proposed that PrP is involved in copper homeostasis. However this has been disputed [40] and it seems unlikely that PrP is implicated in copper trafficking across membranes. Another proposal focuses on PrP moderating oxidative stress. Also this scenario seems in our opinion unlikely as Cu^{2+} is already in its oxidized state. This assumption does not imply that PrP has no moderation effect on other causes of cellular stress.

The binding of metal ions to PrP is not surprising if one takes the nature of intrinsically disordered proteins into account. As discussed above, these structural elements explore conformational space in order to find an appropriate binding partner. Obviously, the N-terminal sequence of PrP is such that it can easily accommodate two-valent cations. Whether these data have functional relevance remains to be proven.

4.2 Interaction of PrP with Small Molecules

The interaction of amyloids with small organic molecules is principally very interesting and important because of their medicinal potentials for treatment of Alzheimer, Parkinson, Creutzfeld-Jakob, or other amyloid related diseases. A few libraries of natural and synthetic compounds have been screened [94–96]. Charveriat et al. [94] identified eight inhibitors of prion replication in vitro. Seven of these small molecules belong to 3-aminosteroids whereas one compound is a derivative of erythromycin A. Screening a library of 10,000 small molecules, 6 compounds displayed an inhibitory effect on PrP^{Sc} propagation in scrapie-infected N2a cells [96]. In the third study pyridine dicarbonitriles were tested [95]. Additionally, bifunctional diketopiperazines have been implicated as antiprion drugs [97]. A different type of compound, so-called chemical aggregators inhibited amyloid formation of yeast and mouse prion proteins [98]. Earlier work had shown that the cationic lipopolyamine DOSPA hindered de novo synthesis of PrP^{Sc} and disrupted preexisting cellular PrP^{Sc} [99].

More interesting results were obtained from the analysis of polyphenols which are ingredients of green tea. Epigallocatechin gallate (EGCG) and gallocatechin gallate induced the transition of mature PrP^{C} into an amorphous aggregate distinct from PrP^{Sc} [100]. In scrapie infected cells application of EGCG stopped PrP^{Sc} formation; however, it interfered with the stress protecting properties of PrP^{C}. In a thorough analysis Ehrnhoefer et al. could show that EGCG inhibits fibrillogenesis of α-synuclein and amyloid-β [101]. Apparently EGCG turned out to be strain selective for yeast PrP Sup35 [102]. A synergistic effect with another small molecule (a methoxyanilino phthalimide) was demonstrated which eliminated diverse Sup35 strains.

Amyloid-binding compounds which are traditionally used in histopathology slowed aggregation in vitro and in cell cultures [103, 104]. In a most recent paper it was demonstrated that one of these dyes (thioflavin T) extended the lifespan of adult *Caenorhabditis elegans* and suppressed β-amyloid toxicity [105]. This finding might lead to new drugs not only related to protein homeostasis also possibly having an impact on other amyloid-forming proteins.

4.3 Interaction of PrP with Peptides

A new development has emerged with the discovery that PrP^{C} is required for neurotoxic effects PrP^{Sc} [106–108]. The intrinsically disordered N-terminus and the C-terminal GPI-anchor were identified to confer this property [109]. Another observation suggested that PrP^{C} may also play a role in Alzheimer disease [110, 111]. It was later recognized that amyloid-β oligomers interact with the N-terminus of recombinant PrP [112]. It was an intriguing idea that amyloid-β binds to PrP^{C} connecting Alzheimer with Creutzfeld-Jakob disease. However, Resenberger et al. [113] demonstrated that PrP^{C} interacts via its N-terminus with other β-forming peptides (among others, yeast prion protein and designed β-peptide), mediating neurotoxic signaling. This interaction of intrinsically disordered proteins with β-sheet proteins seems to be a general phenomenon [114]. This new line of research might lead to a better understanding of the functional properties of PrP^{C} in particular and the role of disordered proteins in general.

5 Concluding Remarks

Chemical biology of prion proteins has revealed important insight into its in vitro properties. It could provide the core structure of PrP and provide evidence for the α-helix to β-sheet transition. The most important developments are those which enable specific modification of proteins in order to obtain tailor made analogs. The implantation of expressed protein ligation (EPL) promises to fill the gap between in vitro and in vivo studies. This gap is obvious if one compares the different lines of research. One has the feeling that, e.g., they live separate lives without communicating with each other. Future cooperation between cell biology and chemical biology will be necessary and fruitful in order to tackle pertinent questions in cell biology.

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The PrP-Like Proteins Shadoo and Doppel

David Westaway, Nathalie Daude, Serene Wohlgemuth, and Paul Harrison

Abstract An almost unique place within protein databases, twenty-five years of study has underscored the enigmatically subtle role of PrP^C in normal cell biology. It seems that PrP has evolved (and survived) to perform a function that does not have a precedent amongst transmembrane cell-surface proteins, perhaps representing a new type of plasma membrane ecosystem. In a context where we await a clarifying insight to unify a panoply of PrP^C data into logical molecular framework, the GPI-anchored N-glycosylated Doppel and Sho proteins are tantalizing in that they correspond roughly to the front and back halves of PrP^C itself. These molecules may be simpler – and more "understandable" – entities that can be pursued in parallel to PrP^C, and could open up the biology of mammalian prion proteins from fresh directions. Dpl has a profound role in successful gametogenesis that warrants close scrutiny and a case for deeper study can be made for Sho, a recently discovered CNS-expressed protein with many parallels to established facets of PrP biochemistry. In an aerial view of biomedical research, Sho and Dpl can be considered as adjacent islands in a prion protein archipelago. As such, the coming years of molecular exploration should be extremely interesting.

Keywords Creutzfeldt–Jakob Disease · Doppel · Neuroprotection · Prion · Shadoo

Contents

D. Westaway (✉), N. Daude, and S. Wohlgemuth
Centre for Prions and Protein Folding Diseases, University of Alberta, Edmonton, AB, Canada
e-mail: david.westaway@ualberta.ca

P. Harrison
Department of Biology, McGill University, Montreal, QC, Canada

1 Introduction

Prion diseases, including the prototype scrapie of sheep and Creutzfeldt–Jakob disease (CJD) of humans, are fatal and incurable neurodegenerative disorders. They most typically manifest as transmissible or infectious diseases caused by a proteinaceous, infectious, etiological agent, the prion protein, PrP. Disease can be inadvertently triggered by clinical procedures, as in the case of iatrogenic CJD caused by prion-contaminated growth hormone, or can be initiated in a laboratory setting by inoculating brain homogenate from affected animals into a recipient (referred to as experimental prion disease). In the case of variant CJD (vCJD), occurrence is inextricably linked to the UK epidemic of bovine spongiform encephalopathy (BSE) and is thought to involve infection by an oral route from BSE-contaminated food. Chronic wasting disease (CWD) represents an emerging prion disease with the strongest propensity for contagion; affecting a number of farmed and feral cervids its spread across the USA and Canada represents a formidable threat for wildlife management, for agricultural activity, and for First Nations communities.

Pathologically, prion diseases are often characterized by spongiform change in the central nervous system (CNS), sometimes accompanied by extracellular amyloid deposits. Highly infectious prion preparations do not contain virion-like structures nor can discrete genome-length nucleic acids be detected within such preparations. Instead many lines of evidence indicate that in the disease process a benign, host-encoded α-helical glycoprotein (PrP^{C}) undergoes a conformational transition to a β-sheet enriched and infectivity-associated form, denoted PrP^{Sc}. This transition is typically marked by reduced detergent solubility and acquisition of resistance to proteinase K digestion in vitro. In strong, independent support of a central role for altered PrP in disease pathogenesis and replication of the transmissible agent, missense mutations in the prion protein structural gene, *Prnp*, causes a modulation

of prion disease phenotypes, while knockout of the mouse *Prnp* gene renders animals completely resistant to experimental prion infections [1]. Similarly, missense mutations in ovine *PRNP* modulate susceptibility to classical and atypical scrapie. In humans, apart from the modulatory effects of missense polymorphisms such as M129V, nonconservative replacements in *PRNP* cause a variety of inherited prion diseases with autosomal dominant presentation. Lastly, beyond infectious and genetic manifestations, human epidemiological studies reveal a further remarkable facet of prion disease, namely a sporadic etiology apparently unrelated to either agent exposure or the cryptic presence of germ-line *PRNP* mutations that are incompletely penetrant. This situation is exemplified by the disease sporadic CJD (sCJD). Moreover, while sCJD was first discerned by epidemiological studies in the 1980s, detailed surveillance of livestock prompted by European and American BSE crises has provided evidence that animal populations (and consequently geographic regions) that were thought to be free of prion disease may be affected with animal versions of sporadic prion disease. Nor98 disease of sheep is a case in point [2]. The reader is referred to other chapters in this "Prion Proteins" volume of Current Chemistry for recent reviews elaborating on the biology and genetics of PrP^{C} and PrP^{Sc} and the cardinal points of prion biology mentioned thus far. The purpose of this review is to present a synopsis of the biology of the two other members of the mammalian prion glycoprotein family, namely Shadoo (Sho) and Doppel (Dpl) (Fig. 1 and Table 1).

The more recently discovered Sho protein bears similarity to two features within PrP: the hydrophobic domain (HD), referred to by some researchers as the central region (CR), and a series of tandem repeats with positively charged residues. Both of these regions in PrP^{C} are considered to be unstructured, or at least less structured than the carboxy-terminal domain. Interest in Sho was catalyzed by the observation that it is expressed predominantly in the CNS and is perpetuated by a number of PrP-like properties, beyond mere sequence homology, and by an intriguing down-regulation phenomenon in prion infected cells. Dpl, discovered several years earlier, bears a resemblance to the carboxyl-terminal globular domain of PrP^{C}. Dpl is expressed in the male reproductive tract and investigations into its biology have centered not only upon reproduction (predictably) but also on the unexpected neurotoxic effects of ectopic Dpl expression in the CNS. Many laboratories have pursued this phenomenon because of (1) its possible significance as an instructive caricature of normal signaling from PrP^{C} and (2) because this form of neurotoxicity is blocked by wild type (wt) PrP^{C} in situ in the CNS and by Sho in primary neuronal cultures [3, 4]; in consequence, this paradigm is described at considerable length in Sect. 3.

With these guiding remarks dispatched, our goal now is to describe the properties of *SPRN* genes (encoding Sho), alleles, expression patterns, proteins, and phenotypes, followed by an analogous point-by-point analysis for *PRND* (encoding Dpl). We then turn to a discussion of shared chemical properties and origins, the potential for shared physiology by way of intersecting biochemical pathways and, finally, speculate on a larger perspective and on promising directions for future research.

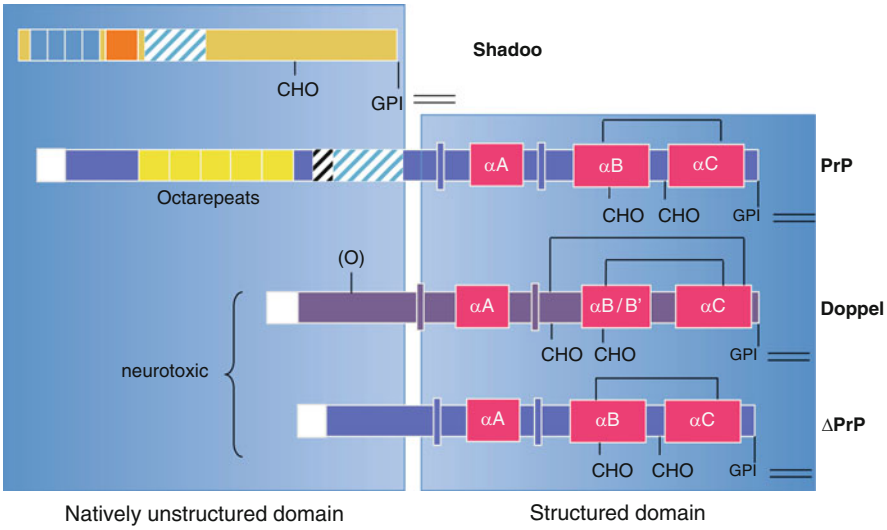

Fig. 1 Domain structure of the PrP protein family. Sho, PrP, Dpl, and internally deleted forms of PrP, noted here, generically, as ΔPrP are shown. The proteins are organized around natively unstructured and α-helical structured domains. The exact boundaries between the domains are approximate. The central hydrophobic tract is indicated by *blue diagonal shading* (see also Fig. 4). For the Sho protein the *blue shaded rectangles* indicate degenerate arginine-glycine-glycine (RGG) repeats. The *orange shaded rectangle* encompasses a different type of arginine-rich tract of the general form RVRVRPAPR. *White boxes* in PrP and Dpl indicate an N-terminal tract of charged residues. The *diagonally shaded box* with *black stripes* indicates a second type of charged patch. CHO indicates N-linked carbohydrates. Disulfide linkages are shown by *black lines*. Alpha helices are shown as *crimson boxes* (αA, αB, and αC) while *narrower boxes* denote β-strands. GPI denotes the glycosylphosphatidyl inositol anchor

Table 1 Properties of PrP, Sho, and Dpl

Property	PrP^C	Sho	Dpl
Tissue with high levels of expression	Brain and many others including, lung, cardiac muscle, circulating lymphocytes	Brain	Male reproductive tract
Natively unstructured region?	N-terminal half of the molecule	Whole protein?	Small N-terminal tract?
α-Helical domain	C-terminal	Not described	C-terminal
Allelic variants linked to human prion disease?	Yes (many)	Yes (two)	Controversial (unlikely)
Refolds to β-sheet form in vitro?	Yes	Yes	No
Refolds to β-sheet form in vivo?	Yes	No	No
Protein level drops during prion disease pathogenesis?	No overt change	Yes	No
Infectious form known?	Yes-PrP^{Sc}	Unknown	No

See also Figs. 1 and 4

2 The *SPRN* Gene and the Sho Protein

2.1 Gene Origins, Duplications and Linkage Groups

Sho was first identified as a genomic open reading frame (ORF), and subsequently in zebrafish ESTs by Premzl et al. [5]. Sho coding sequences have since been found in many fish, amphibian, and mammalian species. The presence of Sho in all the higher mammals suggests physiological utility (although there is one high-coverage mammalian genome assembly, i.e., horse, without the gene [6]). The chromosomal organization surrounding the human, sheep and mouse *Sprn* genes as well as three zebrafish linkage groups are presented in Fig. 2. For a point of comparison, *Prnp*/*Prnd* linkage groups are shown in Fig. 3. In the bovine genome assembly (not shown), the Paox gene (peroxisomal *N*(1)-acetyl-spermine/spermidine oxidase) is inverted compared to the human syntenic group [8]. In mice the gene order is Echs1-Paox-Mtg1-Sprn which is then followed by a cluster of olfactory receptors (Olfr 522–524). Since olfactory receptors and pseudogenes occupy an appreciable portion of the mouse genome, this juxtaposition adjacent to *Sprn* may have no special significance and may merely reflect the requirements of mouse behavioral ecology upon genome usage. On the other hand, PrP^C has been linked to the murine olfactory response [9], perhaps suggesting further investigation into this area. In terms of additional Sho-like genes in mammals, an *SPRN* pseudogene was first noted by Premzl et al. in humans [10]. This is defined by two frameshifts and stop codons and occurs in the context of a segmental duplication. This duplication is found in multiple primates (human, chimpanzee, macaque, orangutan, galago, and tarsier [6]). In humans, the pseudogene (*SPRNP1*) is transcribed and based upon (1) an overlap with the SYCE gene encoding a protein component of the synaptonemal complex and (2) a possibility of transcriptional interference with SYCE; Harrison et al. speculate that *SPRNP1* might impact the control of meiosis.

Fish have undergone chromosomal events that have manifested in multiple copies or paralogs of genes that are single-copy in mammalian species [11]. In the specific instance of Sho, many fish (zebrafish, fugu, and tetraodon) have at least two Sho genes [5, 6, 10, 12] (Fig. 2). A parallel situation exists for PrP itself in fish, with 1–4 PrP-like genes being represented in different species' genomes [6, 12], although it should be noted that the globular domain of these fish PrPs may be quite distinct from the familiar mammalian prototypes, having multiple large loops that are predicted to have protein disorder [13]. Dpl genes are present in fewer species than *PRNP* and *SPRN* (for example, they are not present in birds and fish) and may simply represent a more recent evolutionary arrival, as discussed below in Sect. 3. This then raises questions about the ancient evolutionary origins of the "remaining" genes, i.e., *SPRN* and *PRNP*, and the nature of the founding member of the prion protein family.

While an approach to ontogeny through compiling gene inventories in current species is a practical start to this problem, the assumptions for this strategy have to be weighed against the possibility of changing selective pressures. For example, as

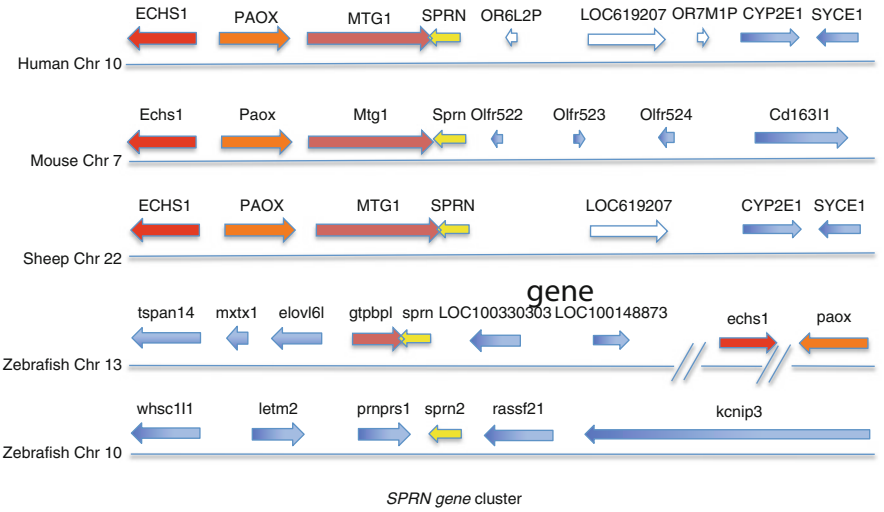

Fig. 2 Linkage groups for *Sprn* genes in different organisms. Linkage groups for different organisms, with corresponding chromosomal localizations. *White arrows* represent pseudogenes. *Human chromosome 10* (NCBI Map Viewer Build 37.2): ECHS1, enoyl CoA hydratase, short chain, 1, mitochondrial; PAOX, polyamine oxidase (exo-N4-amino); MTG1, mitochondrial GTPase 1 homolog (*Saccharomyces cerevisiae*); SPRN, shadow of prion protein homolog (zebrafish); OR6L2P, olfactory receptor, family 6, subfamily L, member 2 pseudogene; LOC619207, scavenger receptor protein family member (pseudogene); OR7M1P, olfactory receptor, family 7, subfamily M, member 1 pseudogene; CYP2E1, cytochrome P450, family 2, subfamily E, polypeptide 1; SYCE1, synaptonemal complex central element protein 1. *Mouse chromosome 7* (NCBI Map Viewer Build 37.2): Echs1, enoyl Coenzyme A hydratase, short chain, 1, mitochondrial; Paox, polyamine oxidase (exo-N4-amino); Mtg1, mitochondrial GTPase 1 homolog (*S. cerevisiae*); Sprn, shadow of prion protein; Olfr522, olfactory receptor 522; Olfr523, olfactory receptor 523; Olfr524, olfactory receptor 524; Cd163l1: CD163 molecule-like 1. *Sheep chromosome 22* (from [7]): ECHS1, enoyl CoA hydratase, short chain, 1, mitochondrial; PAOX, polyamine oxidase (exo-N4-amino); MTG1, mitochondrial GTPase 1 homolog (*S. cerevisiae*); SPRN, shadow of prion protein homolog (zebrafish); LOC619207, scavenger receptor protein family member (pseudogene); CYP2E1, cytochrome P450, family 2, subfamily E, polypeptide 1; SYCE1, synaptonemal complex central element protein 1. *Zebrafish chromosome 13* (NCBI Map Viewer Zv8): tspan14, tetraspanin 14; mxtx1, mix-type homeobox gene 1; elovl6l, ELOVL family member 6, elongation of long chain fatty acids like; gtpbpl, GTP binding protein like; sprn, shadow of prion protein; LOC100330303, visceral mesodermal armadillo-repeats-like; LOC100148873, hypothetical LOC100148873. *Zebrafish chromosome 10* (NCBI Map Viewer Zv8): Whsc1l1, Wolf-Hirschhorn syndrome candidate 1-like 1; letm2, leucine zipper-EF-hand containing TM protein 2; prnprs1, prion protein, related sequence 1; sprn2, shadow of prion protein 2; rassf2l, Ras association (RalGDS/AF-6) domain family 2, like; kcnip3, Kv channel interacting protein 3, calsenilin

mentioned, there is one recently sequenced, high-coverage genome assembly that appears to lack *SPRN* genes (horse) and, unexpectedly, further PrP family members were observed in the reptile *Anolis caroliensis* [6]. A scenario wherein PrP was the progenitor gene, giving rise to both Dpl and Sho, does not necessarily fit with the occurrence of Sho in multiple phyla but has been considered in recent work by

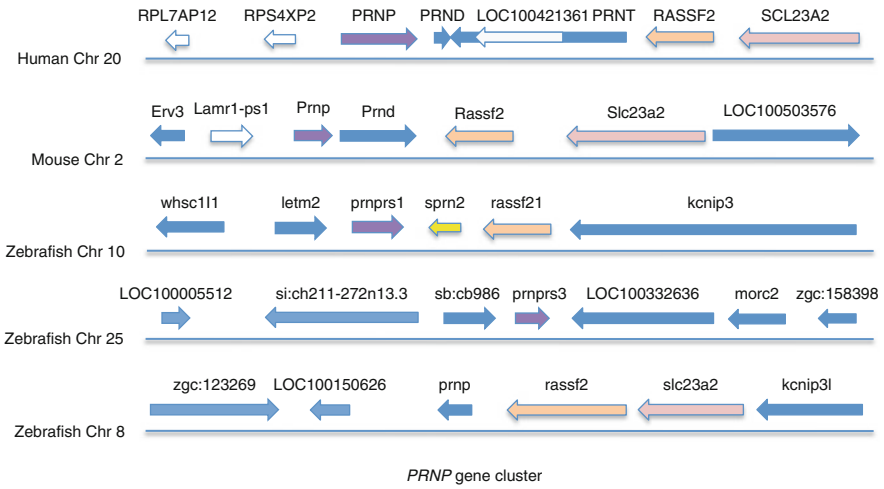

Fig. 3 Linkage groups for *Prnp* genes in different organisms. Linkage groups for different organisms, with corresponding chromosomal localizations. *White arrows* represent pseudogenes. *Human chromosome 20* (NCBI Map Viewer Build 37.2): RPL7AP12, ribosomal protein L7a pseudogene 12; RPS4XP2, ribosomal protein S4X pseudogene 2; PRNP, prion protein; PRND, prion protein 2 (doublet); PRNT, prion protein (testis specific); LOC100421361, isopentenyl-diphosphate delta isomerase 1 pseudogene; RASSF2, Ras association (RalGDS/AF-6) domain family member 2; SLC23A2, solute carrier family 23 (nucleobase transporters), member 2. *Mouse chromosome 2* (NCBI Map Viewer Build 37.2): Erv3, endogenous retroviral sequence 3; Lamr1-ps1, laminin receptor 1 (ribosomal protein SA), pseudogene 1; Prnp, prion protein; Prnd, prion protein doublet; Rassf2, Ras association (RalGDS/AF-6) domain family member 2; Slc23a2, solute carrier family 23 (nucleobase transporters), member 2; LOC100503576, hypothetical LOC100503576. *Zebrafish chromosome 10* (NCBI Map Viewer Zv8): Whsc1l1, Wolf-Hirschhorn syndrome candidate 1-like 1; letm2, leucine zipper-EF-hand containing transmembrane protein 2; prnprs1, prion protein, related sequence 1; sprn2, shadow of prion protein 2; rassf2l, Ras association (RalGDS/AF-6) domain family 2, like; kcnip3, Kv channel interacting protein 3, calsenilin. *Zebrafish chromosome 25* (NCBI Map Viewer Zv8): LOC100005512, C-C chemokine receptor type 2-like; si:ch211-272n13.3, si:ch211-272n13.3; sb:cb986, sb:cb986; prnprs3, prion protein, related sequence 3; LOC100332636, hypothetical protein LOC100332636; morc2, MORC family CW-type zinc finger 2; zgc:158398, zgc:158398. *Zebrafish chromosome 8* (NCBI Map Viewer Zv8): zgc:123269, zgc:123269; LOC100150626, hypothetical LOC100150626; prnp, prion protein; rassf2, Ras association (RalGDS/AF-6) domain family member 2; slc23a2, solute carrier family 23 (nucleobase transporters), member 2; kcnip3l, Kv channel interacting protein 3, calsenilin, like

Schmitt-Ulms et al. into the ancient origins of prion genes [14]. Their analysis focused on a particular part of PrP – the globular domain – in a scenario that may reflect "the progenitor of a progenitor." PrP^C interacting partners, retrieved by a tagging and crosslinking method, contained, amongst other interactor proteins, two solute carrier proteins. These proteins were ZIP10, aka Slc39A10 and ZIP6, aka Slc39A6, both being putative zinc transporters. ZIPs are part of a large family of solute transporters. They have a polytopic multitransmembrane (TM) domain organization but glimpses of their biological activity are only just coming into view (reviewed by Ehsani et al. [15]). Remarkably, bioinformatic interrogation of

these proteins predicted a PrP-like fold in the ectodomain region, N-terminal to the TM helices, which line the solvent exposed core of these selective ion channels. This has led to the hypothesis that PrP genes had their origins in retrotransposition. This evolutionary route to present day PrP-family genes would involve two clear steps: (1) reverse transcription of a messenger RNA from a progenitor of the Zip5/6/10 gene family, with reintegration at a distal chromosomal site to give modern day intronless PrP coding sequences and (2) subsequent acquisition of noncoding exons during metazoan evolution. Such a duplication route via retrotransposition has resulted in hundreds of retrogenes in rodents and primates during mammalian speciation [16] but would have occurred for PrP early in (or earlier than) vertebrate speciation. It is speculated that mutation in the vicinity of a (former) TM helix gave rise to a hydrophobic sequence that functions as a consensus glycosylphosphatidylinositol (GPI) attachment site in the PrP genes found in contemporary vertebrates. A structural homology correlate of the idea that ZIP proteins were a progenitor to a primordial PrP-like molecule has been pursued to date by threading algorithms and reveals a common "fold" encompassing helices A, B, and C, and two beta strands lying at a ~90° angle to helices B and C [14]. Obtaining the NMR or crystallographic structure of amphibian, fish and mammalian ZIP ectodomain regions will be of considerable importance to extend these findings. However, as interesting as these concepts are, a hypothesis for the origin of a primordial PrP gene does not necessarily address the origin of a primordial Sho protein. The options to be considered for *SPRN* are:

1. A genome rearrangement event, independent of the event leading to the primordial PrP gene, led to Sho coding sequences emerging from the N-terminal coding region of an ancient ZIP gene.
2. A primordial PrP gene evolved from ZIP genes and acquired a Sho-like region de novo. This is plausible since Sho is comprised mainly of repetitive or low-complexity sequence. A duplication of the primordial PrP gene then suffered loss of the globular PrP domain to yield the present-day consensus form of the Sho genes. Another later gene duplication event from PrP gave rise to Dpl. This perspective certainly deviates from prior analyses that place Sho at the head of the family tree [10] for the reasons given below.
3. A primordial PrP gene evolved from ZIP genes and inserted into a "spare" copy of Sho (generated by duplication of a Sho progenitor) at a C-terminal site.
4. Sho arose completely independently of PrP, perhaps at an earlier stage in evolution [10], and perhaps also completely independently of ZIPs. Accordingly, present-day similarities would be the result of convergent evolution.

Scenario 1 remains plausible but cannot be resolved given the current known cohort of ZIP and PrP family sequences. The key evidence for resolving these scenarios comes from the conservation of adjacent genes [10]. In mice, the *Sprn* gene and the prion gene complex [17], comprised of *Prnp* and *Prnd* (and, in at least 16 mammalian species, *PRNT* [6, 18]) are on different chromosomes (Figs. 2 and 3) but, interestingly, some evidence for linkage can be found in Fugu and zebrafish. Here the *prnps1* gene (which contains a PrP globular C-terminal domain) and

a second copy of *Sprn*, *sprn2*, lie directly adjacent, albeit in opposite transcriptional orientations (Fig. 2), suggestive of a duplication of a progenitor cellular prion protein followed by an interstitial chromosomal inversion. Since the most similar area between PrP and Sho proteins is the HD, these adjacent genes and their flanking genes that have sometimes survived into diverse phyla (adjacent to both *SPRN* and *PRNP*, e.g., Rassf2 [10]) provide evidence for intertwined origin (scenarios 1–3) rather than a pathway of convergent evolution (scenario 4).

In these considerations, a complication is added by the existence of transcripts spliced together from exons in different, adjacent genes, i.e., the so-called "chimeric transcripts" that can be derived from read-through from adjacent gene transcription units in vertebrates. These loci include *Prnp*/*Prnd* in mammals [19]. Formation of chimeric transcripts provides a mechanism for merging of adjacent genes after an initial genome rearrangement event. For scenario 3 above, genome rearrangement via retrotransposition of at least part of a ZIP gene progenitor to a genomic position adjacent to an ancestral Sho gene may have occurred; this may have been followed by subsequent merging of the two genes via formation of chimeric transcripts or via some other local rearrangement event to yield the present day PrP sequences. More generally, in all of the above reflections, the complexity of genome rearrangement events (e.g., a spectrum including retro-transcription and integration, fused mRNAs, and the possibility of horizontal gene transfer and virally-mediated gene transduction) may temper or confound unequivocal insights into the genetic origin of contemporary PrP and PrP-like proteins.

2.2 *SPRN Coding Sequences and Polymorphic Variation*

2.2.1 *Sprn* in Mouse

Besides the aforementioned *a–c* alleles of mouse *Prnp* (and other haplotypes *d–f* reflecting noncoding polymorphisms [20–22] defining flanking sequence variation), several quantitative trait loci (QTLs) for prion susceptibility were found to influence scrapie incubation time [23–27] in cattle [28, 29] and in sheep [30, 31]. Two have now been defined as HECTD2 and Cpne8 [32, 33] but, interestingly, the D7Mit259 marker found adjacent to a QTL by Stephenson et al. [23] is within 5 Mb of *Sprn*. All the other QTLs found in mice, sheep, and cows were nonsyntenic for *Sprn*. Analysis of 51 inbred strains of *Mus musculus*, as well as of *Mus spretus*, has shown little in the way of polymorphic variation in *Sprn* that could suggest an alteration in biological activity germane to prion disease pathogenesis [34–36]. Thus most mouse lineages examined did not present any polymorphisms and were similar to the published sequences (e.g., GI: 56118240).[1]

[1]In terms of silent polymorphisms, an L11L change was described in SEG/Pas mice, G108G in CAST/Ei, MAI/Pas, PWK/Pas, and SEG/Pas mice, and G136G in SEG/Pas mice. Conservative missense changes include R86K in wood mouse [34–36]. Of these, L134F can be noted to be in the GPI attachment signal peptide.

2.2.2 *SPRN* in Sheep

Sheep have the most allelic variation amongst mammalian species studied to date [35–37]. There is a common polymorphism, V71A, and a common silent polymorphism, Y112Y. Some rare polymorphisms have also been described (G21S, A53P, A65A, E89K, H126P, G132G). Interestingly there are also a number of indel variations: −12, +3, +6, and +9 occurring within the HD, discussed in greater detail below. As noted previously, the abundant coding sequence variation in *SPRN* is strikingly reminiscent of the diversity of ovine *PRNP* alleles first documented close to 20 years ago [38]. Beyond coding sequences, *SPRN* 5′ flanking sequences have also been analyzed by Lampo et al. [37], prompted in part by a hypothesis that *SPRN* and *PRNP* mRNA levels are positively correlated.

2.2.3 *SPRN* in Humans

Analysis of normal human populations (i.e., for the purposes of this discussion, not affected with neurodegenerative disease) has revealed little in the way of *SPRN* polymorphisms [35, 39]. There is a common A to G change, 11 nucleotides prior to the ATG. There is a common coding variant change, T7M, in the signal peptide of the protein and a silent mutation (T to C change, G61G). Echoing results found in sheep (below), indels of Ala-Ala-Ala-Gly repeats, albeit rare, were also found [35, 39]. These data point to an analogy with the human *PRNP* locus where, besides the disease-modulating M129V variation, there are a paucity of missense variations (these principally comprising G54S, a deletion of one octarepeat, G142S, N171S, V209M, and E129K), all with low or nonexistent pathogenic potential [40].

2.2.4 Variation in the Sho HD in Mammalian *SPRN* Genes

In PrP, the central HD is extremely conserved (11 differences in 130 species) to the extent that the first non-mammalian PrP gene (chicken) to be identified was retrieved via sequence homology in this region [41]. The hydrophobic region of Sho shows the most homology with PrP – indeed, it led to the identification of Sho – and, as in PrP, is remarkably conserved in full-length copies of the gene. Given the overall degree of conservation of the Sho HD it is therefore of considerable interest to have found deletion and insertion variants in this region, in both sheep and bovine species and, more rarely, in humans [35, 36] (Fig. 4). These genetic variations may arise because mammalian coding sequences for the Sho HD are arranged as tandem repeats, a situation slightly different from the PrP HD, which is palindromic. Thus our own analyses have identified and denoted the repeats as R1–R5, corresponding to amino acid sequences AAAG-AAAG-AAAG-VAAG-LAAG [35]. As with other tandem repeats (and reminiscent of – though perhaps not functionally equivalent to – PrP octarepeat expansions), unequal crossing-over

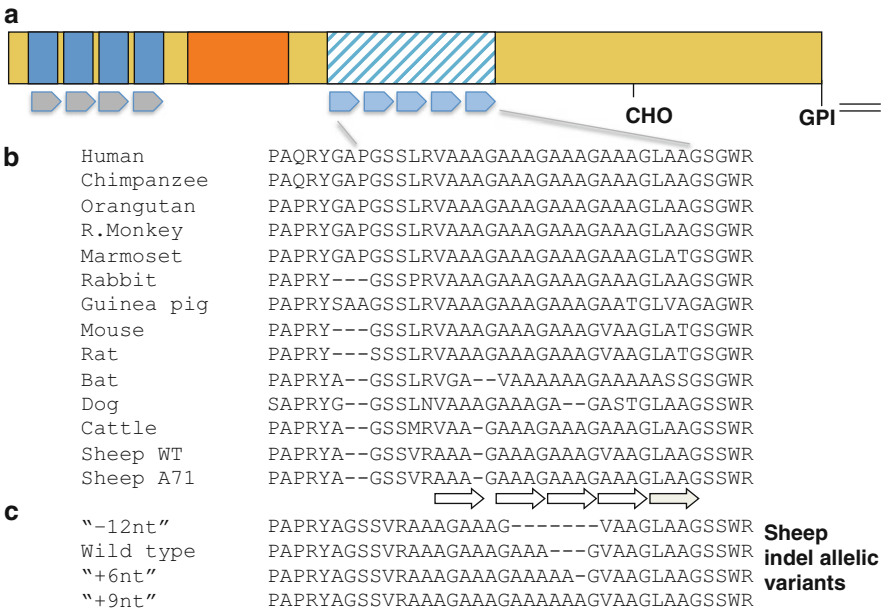

Fig. 4 Tandem repeats and the structure of the Sho protein. (**a**) Domain structure of the Sho protein. *Blue shaded rectangles* indicate degenerate arginine-glycine-glycine (RGG) repeats with tandem head-to-tail organization indicated by *gray arrows*. An *orange shaded rectangle* encompasses a different type of arginine-rich tract of the general form RVRVRPAPR. A central hydrophobic tract is indicated by diagonal shading and is itself made up of tandem repeats composed of four amino acids (*blue arrows*). (**b**) Expanded view and sequence alignment of the hydrophobic tract from different species to demonstrate tandem repeat organization. (**c**) Insertion/deletion (indel) allelic variants of the hydrophobic tract seen in sheep. Similar, albeit rare, indel variants are also described in humans (not shown). Scale is approximate

during DNA replication can lead to deletion or insertion of the repeat units. In sheep, alleles representing these rearrangements can reach frequencies of 20%. The rearrangements often involve changes in or adjacent to R3, with R4 and R5 presumably being spared because their first codon is not an alanine (as per R1–R3). Interestingly, a trinucleotide insertion in sheep to create an extra residue at codon 65 makes a change within the tandem repeat structure to create a replica of the canonical PrP palindromic amino acid sequence AGAAAAGA [36]. In this respect this allele resembles the Sho-1 and Sho-2 proteins of carp and zebrafish, which also deviate from the R1–R5 HD coding sequence organization (Fig. 4). It is to be noted that the PrP palindromic HD region is proposed to form a binding site for protein–protein interactions and may comprise an interface between normal PrP and its disease-specific isoform, PrP^{Sc} (see below). Thus it will be interesting to see if this mammalian Sho allele has acquired biological properties that overlap the properties typically equated with mammalian PrP (i.e., serving as a precursor to PrP^{Sc} and/or protection against neurotoxic insults).

2.3 Expression of the Sprn Gene

Sprn is clearly expressed in CNS neurons and in situ hybridization and some studies with polyclonal antisera support a pattern of expression that overlaps with that of *Prnp*, albeit a more restricted pattern. Prompted by this information and by an interest in the processes of prion disease pathogenesis, *Sprn* mRNA and protein expression have been investigated in the brains of prion-infected rodents. These analyses have yielded the unpredicted result that the mature Sho protein is drastically down-regulated in later stage disease [4, 42] whereas cognate mRNA levels are unaltered or even mildly elevated [34]. Subsequent analyses to probe deeper into this effect have again failed to define abnormalities in *Sprn* mRNA expression but have instead pinpointed a novel and previously cryptic regulatory mechanism; a proteostatic effect that couples degradation of the mature Sho glycoprotein to the in vivo accumulation of protease-resistant PrP (Genovesi et al., Watts et al., both in preparation). These findings may define a regulatory mechanism that applies to misfolded GPI-linked proteins, thereby extending recent insights into the clearance of misfolded polytopic (multi-TM) plasma membrane proteins [43].

2.4 Sho Structure and Protein Partners

Structural features of Sho are presented in Fig. 1 and, along with potential binding partners, have been the subject of a recent review [44]. In synopsis, the molecule can be said to be PrP-like in terms of having (1) charged tandem N-terminal repeats, (2) a central HD, (3) a distinct C-terminal region decorated by N-glycosylation, (4) cell-surface presentation via GPI anchor, (5) a number of shared protein partners identified by interactome analysis in transfected N2a neuroblastoma cells, and (6) an ability to refold in vitro into an amyloid-like form. Conversely, the features that diverge from PrP are that (1) the N-terminal repeats do not resemble histidine-containing, copper-binding octarepeats but contain "RGG" boxes that have been associated with RNA binding, (2) the HD is encoded as five tandem repeats of a canonical four amino acid sequence to thereby encompass "GxxxG" motifs, whereas the analogous area of PrP includes a palindrome AGAAAAGA and (3) the C-terminal region is not known as yet to encompass any helices whereas in PrP there is a 3-helix bundle structure. In tissue culture and in adult brain both Sho and PrP engender membrane-anchored "C1" species that derive from cleavage near the midpoint of the molecule but it is currently unknown whether a common protease processes both proteins. Relationships between PrP^C and Sho are summarized in Table 1.

2.5 Towards an Understanding of Sho's Biological Functions

Without pre empting the complete discussion of the push–pull experimental paradigm whereby PrP^C and Dpl "compete" to determine neurodegeneration in CNS

neurons (see Sect. 3.7), it can be noted here that Sho has a PrP^C-like neuroprotective effect as measured in primary cultures of cerebellar granule neurons (CGN) [4]. Interestingly, a recent study by Sakthivelu et al. has confirmed and extended aspects of this concept. Specifically, in SH-SY5Y neuroblastoma cells both wt PrP^C and wt Sho had a protective effect against an internally deleted PrP ("ΔHDPrP," partially analogous to Dpl in terms of domain structure). Furthermore, both wt PrP^C and wt Sho had a protective effect against an acute dose of glutamate applied to SH-SY5Y cells [3]. These data beg the larger question of whether Sho, in a CNS context, can also protect against neurotoxic insults, as this certainly seems to be the cumulative case for PrP^C [45]. A divergent concept arising from the same push–pull experimental paradigm is the existence of a hypothetical "back-up" PrP-like molecule called pi (π) [46], this also applying in the context of the intact CNS rather than cultured cells. Given data summarized in Table 1, Sho is currently a plausible candidate for pi. ZIPs [14], though newer on the scene, might also be considered in this role. In an original formulation, overlapping trophic functions of PrP and pi would account for the lack of phenotype in individual knock-out (k/o) animals [46], but inter-crossing for null alleles can be hypothesized to result in a synthetic lethal phenotype. In present day terms, this can be reframed into two questions. First, what is the phenotype of a Sho k/o ($Sprn^{0/0}$) mouse? Here, the functional redundancy hypothesis would predict no basal phenotype in animals housed under normal conditions. Second, what is the phenotype of an $Sprn^{0/0}$ + $Prnp^{0/0}$ double k/o mouse? Here, the functional redundancy hypothesis would predict a synthetic lethal phenotype arising from the loss of CNS neurons. While full answers to these questions are emerging from use of a fully penetrant null allele of *Sprn* (Daude, Carlson et al., in preparation), a preliminary answer has emerged from transient viral knockdown of *Sprn* transcripts within the embryos of $Prnp^{0/0}$ mice. An embryonic lethal phenotype results from this manipulation [47] and suggests that further travel down this avenue of investigation will prove fruitful.

3 The *Prnd* Gene and Its Protein Product, Doppel

3.1 PRND Gene Origins, Duplications and Linkage Groups

Dpl (gene symbol *PRND*) is a divergent paralog of PrP (exhibiting, on average, ~25% sequence identity). Dpl is located downstream of the *Prnp* transcriptional unit in mammals [19, 48]. In terms of origins, this topic was touched on above (Sect. 2.1) and recent evolutionary analyses indicate that Dpl formed from a local duplication of PrP in an early *Tetrapoda* ancestor and was subsequently lost at some point during separation of the bird lineage from reptiles [49]. The detection of at least two further local duplications of PrP in the Anole lizard raises the possibility of a more complicated evolutionary history; these additional PrP family members in Anole cannot be aged accurately until further reptile genomic sequences are

available but they obviously result from more recent local duplications and point towards neo- and subfunctionalizations of PrP^C within reptiles, in a manner not yet observed in other *Tetrapoda*.

3.2 *PRND* Coding Sequences and Polymorphic Variation

3.2.1 Ovine *PRND*: Coselection with *PRNP*?

Linkage disequilibrium for polymorphic markers was found between the ovine *PRND* and *PRNP* loci. Insofar as the genes are immediately adjacent to one another this is perhaps not so remarkable but, in any event, *PRND* was found to be monomorphic in the *PRNP* "ARR/ARR" genotype associated with resistance to the classical form of natural scrapie, whereas a higher frequency of *PRND* heterozygotes (G/A) for a silent substitution in codon 26 was associated with an ARQ/AHQ genotype [50]. The authors speculated that since (1) synonymous substitutions are not always "silent" (for example, they can affect mRNA folding) and (2) Dpl plays a role in fertility, this phenomenon suggests a mechanism whereby sensitivity to prion infections might be inadvertently co-selected with breeding vigor of sheep stocks [51]. In the *PRNP*/*PRND*/*PRNT* linkage group the coding sequences for *PRND* are located downstream of *PRNP* and can be joined – in specialized circumstances arising from genetic engineering – to read-through transcription and splicing from the *PRNP* promoter, which can then cause neurodegeneration, as elaborated upon in Sect. 3.7. On the other hand, there is no clear evidence for a reciprocal effect wherein sequences within the *PRND* transcription unit, which is active primarily in the male reproductive tract, can influence the expression of the *PRNP* gene. Consequently, it is perhaps not so surprising that the attempts to correlate *PRND* polymorphisms with prion disease susceptibility have been unrewarding or have yielded contradictory results, as summarized below.

3.2.2 *PRND* in Livestock

Three polymorphisms (R50H, N110H, and R132Q) have been identified in the *Prnd* gene of cattle and two polymorphisms (I121I, A26A) in sheep [52]. None of these, however, are significantly associated with BSE or scrapie. In another study, DNA polymorphisms of the bovine PRND gene were analyzed in four major breeds – Schwarzbunt (Holstein Friesian), Rotbunt (Holstein Red), Fleckvieh (Simmental), and Braunvieh (Swiss Brown) – resulting in the isolation of the previously known polymorphisms R50H and R132Q and two novel synonymous single nucleotide polymorphisms (SNPs) – C4820T and A5063T. Comparative genotype and haplotype analysis of BSE and control animals revealed a significantly different distribution of polymorphisms C4815T and R132Q in Fleckvieh animals but not in the

other breeds tested. No association to BSE susceptibility was detectable for polymorphisms R50H and A5063T [53].

3.2.3 *Human PRND*

This gene includes at least four reported polymorphisms, with one being very common (M174T), occurring in 48% of the population [54]. As assessed by four groups, M174T has no significant prion disease association [54–57]. However, Schröder et al. [58] reported a statistically significant difference in the distribution of the M174T polymorphism between CJD patients and controls. As reported by Croes et al., while M174/M174 homozygotes were overrepresented in sCJD patients, a meta-analysis on the M174T polymorphism did not show a consistent effect across studies, thus raising the question as to whether this polymorphism is causally related to CJD or whether a *PRND* M174 haplotype is merely in linkage disequilibrium with another (cryptic) polymorphism predisposing to CJD [59]. Another point to bear in mind here is that residue 174 is not in the mature Dpl protein, but in the signal peptide for the GPI anchor. In other studies, a polymorphism at 3′ UTR +28 of *PRND* was associated with sCJD in the Korean population [60]. Again, as Dpl is not notably expressed in the CNS (see Sect. 3.3) and as there is no obvious way wherein *PRND* transcripts might affect "upstream" transcription of *PRNP*, it seems that the explanation supplied by Croes et al. for M174 and disease association might equally well be applied here.

3.3 *Anatomical Expression of the PRND Gene*

Dpl expression has been documented in many mammals including rodents, humans, sheep, goats, and cattle [52, 61–64]. The bulk of the data on gene expression can be grouped into two main themes – expression in the male reproductive tract (the highest site of expression) and expression in the astrocytes of wt animals associated with neoplasia. These anatomical sites will be considered in greater detail below in concert with the phenotypic correlates of this expression but, beyond these, some forms of systemic expression have also been described – for example in splenocytes, as detected by polyclonal antibodies [65, 66] and by amplification of mRNAs, in spleen, heart, bone marrow, and skeletal muscle [67]. Based in part upon these findings and use of a polyclonal α-Dpl antiserum, Cordier-Dirikoc et al. have gone on to report the binding of fluorescently labeled recombinant Dpl (and PrP) to mouse splenocytes and the consequences of such binding on the modulation of Ca^{2+} fluxes [66].

Expression of Dpl in the brain is not considered the norm and consequently the type of expression found by read-through from the *Prnp* promoter in certain $Prnp^{0/0}$ mice is typically described as "ectopic," although there are some literature references to brain expression in wt animals. Dpl expression is described in brain blood

vessel of embryonic mice [68] and immunoreactivity is reported in the dystrophic neurites of senile plaques in Alzheimer's disease [69]. Rather, in accord with studies of *PRND* polymorphisms, there have been no data to suggest that (the very low level of) Dpl expression is modified in scrapie-infected cells and in the brains of patients with CJD [70]. Similarly, expression of Dpl in the CNS of mice does not modulate transmissible spongiform encephalopathy disease [71].

Another form of Dpl expression that can be considered "ectopic" relates to cancer. Astrocytomas comprise a recurring theme in this Dpl/oncology link. Comincini and coworkers investigated *PRNP* and *PRND* expression profiles by real-time reverse transcription-quantitative PCR in low- and high-grade astrocytomas, in glioblastoma-derived cell lines, and in nonglial tumor specimens. *PRNP* expression was scored positive in all the tumoral samples studied but, unlike the nonmalignant samples, *PRND* was aberrantly expressed in glioblastoma multiforme and in two glioblastoma multiforme-derived cell lines. Moreover, *PRND* expression was directly related to the malignancy index of the tumor and was associated with a cytoplasmic immunohistochemical signal [72]. Another report continued this theme with the description of a ~42 kDa form of Dpl that was PNGase-F resistant [73] (unlike normal forms of mature Dpl protein which migrate at about 37 kDa and are PNGase-F sensitive [74]; see Sect. 3.4) but, since only one polyclonal antibody was used for these studies, verification by a different reagent would be useful. While a similar concern pertains to a subsequent study [75] and a study of acute myeloid leukemia cells with a 55–60 kDa Dpl species [76], this criticism cannot be applied to studies described by Sbalchiero et al., who used an anti-Dpl monoclonal D7C7 and fluorescent fusion proteins to follow intracellular sites of Dpl accumulation [77]. A 2007 study used real-time reverse-transcriptase coupled PCR to follow *PRND* expression in a tumor series using target *GAPDH* (control) and *PRND* amplicons [78] but aspects of the analysis deviate from the MIQE guidelines [79] and hence temper a full consideration. When appraising these nascent results it is useful to bear in mind that PrP^C has also emerged into the world of cancer biology [80] in the guise that it can confer resistance to cell death which might otherwise control neoplasia-prone cell populations. This idea is also in broad agreement with the concept that PrP^C has a positive effect upon CNS maintenance. However, as with mainstream CNS expression [81], there is a concern as to how much faith to place in "variant" cytoplasmic isoforms of GPI-linked PrP and Dpl that are posited to modulate intracellular signaling cascades that lead to oncogenic endpoints. On the other hand, repeat sightings of perturbed *PRND* (and *PRNP*) expression in unbiased analyses of oncogenic samples would go a long way towards dispelling doubts and thereby cementing a role in unregulated cell division or cell adhesion processes.

3.4 Structure of the Dpl Protein

The mouse Dpl precursor protein consists of 179 amino acid residues and encompasses a 23 residue N-terminal signal peptide and a 25 residue C-terminal signal

peptide for GPI attachment. There are two N-linked glycosylation sites, at residues Asn99 and Asn111, and two disulfide bonds, with the first being between Cys 109 and Cys 143 and the second between Cys 95 and Cys148 [74, 82]. In addition an O-glycosylation event has been reported for human Dpl in the testis [83] but this result was not verified in cultured cells [84]. Within mammalian Dpl the Cys109–Cys143 linkage is analogous to PrP's Cys178–Cys 213 bond. While Dpl exhibits no more than 25% amino acid identity with PrP in linear alignments [19, 68], an NMR structure shows remarkable similarity to the C-terminal part of PrP in a folded structure encompassing three α helices and two antiparallel β-strands [85–88]. Removal of the Cys95–Cys148 bond by genetic engineering reduces the stability of the folded Dpl protein to urea denaturation [89] and, in a partially reciprocal manner, PrP^{C} can be engineered to have an extra disulfide linkage Cys166–Cys211 without impairing the global fold of the molecule [90], with the resulting molecule being more resistant to chemical denaturation [91]. Parenthetically, the existence of an unpaired cysteine that could form intermolecular disulfides in the Anole lizard version of Dpl provides a clue to a possible multimeric functional Dpl protein complex [49] (see Sect. 3.4); however, mammalian Dpl has been shown not to interact with itself in a yeast two-hybrid assay [92].

In vitro Dpl is able to bind copper as shown by a variety of methods, including a novel "footprinting" protocol to protect participating histidine residues against diethyl pyrocarbonate modification [93]. By electron paramagnetic resonance and fluorescence spectroscopy, the in vitro binding of copper(II) to human recombinant Dpl occurs with a different stoichiometry from that observed for recombinant PrP [94], a finding that is compatible with Dpl having a short N-terminal region without tandem octarepeats and a second type of histidine-containing copper binding motifs that are present in PrP. Instead, Dpl's metal binding activity maps to a subdomain within the helical region. Copper(II) complexes can form complexes with peptide fragments encompassing the sequence 122–130 of the human Dpl protein [95], such that a Dpl α-helix peptide fragment can mimic the copper(II) interactions seen with the full-length protein [96].

These data lead to an appreciation of the specialized metal-binding properties of sequences within PrP's N-terminal region, which has remarkable binding capabilities, extending over five orders of magnitude [97], and suggest that one possible mechanism for the antagonistic activity of PrP/Dpl would involve competitive metal-binding. At the same time the data raise the question of a different type of specialization/function of the short tract of amino acids in Dpl that are not involved in the NMR solution structure, i.e., residues N-terminal to arginine 51 of the mouse sequence. Residues 1–26 of the mouse Dpl protein comprise the signal peptide (SP) but, based upon the concept of cytosolic forms of PrP that retain the SP [98], a synthetic Dpl peptide fragment of 1–30 was assessed in the presence of phospholipid and detergent micelles [99]. The peptide was capable of adopting a TM topology and of forming pores, possibly germane to toxic activities seen in some cellular assays. As to the function of residues 27–50 in the mature Dpl protein formed after SP cleavage, our own studies suggest interaction with an endoprotease

to create "cryptic" endoproteolytic products, perhaps being analogous to metabolically stable fragments of PrP and Sho (Mays et al., in preparation).

Beyond the important task of apportioning discrete roles to Dpl and PrP^C, some chemical studies of recombinant protein have gone further, to appraise the properties of a chimeric polypeptide composed of the N-terminal "unstructured" domain of PrP^C (residues 23–125) and the α-helical domain of Dpl (residues 58–157). This chimeric protein displays a property described as PrP-like, insofar as exposure to high salt results in the formation of protein oligomers with partial resistance to proteolytic digestion in vitro [100]. The significance of this finding in a biological context is currently unclear though, for two reasons. First, the most notable toxic property of Dpl occurs when it is expressed in the CNS yet this event is not associated with an aggregation process and/or the generation of protease-resistant forms of Dpl protein. Second, as described below, the in vivo effect of the wt PrP N-terminal region fused to the α-helical domain of Dpl is to suppress pathogenic potential in the CNS.

Any discussion of protein folding in the prion field would be incomplete without a consideration of *mis*folding to disease-associated isoforms. In this regard there is no evidence to suggest that Dpl can be driven into a β-sheet enriched pathogenic "Dpl^{Sc}" form, and $Prnd^{0/0}$ mice lacking the protein are perfectly capable of sustaining a prion infection.

3.5 Cellular Environments and Protein Partners of the Dpl Protein

Dpl, like PrP^C, is attached to the plasma membrane (PM) by a GPI anchor [19, 74]. Consequently it is plausible that when both proteins are coexpressed in the male genital tract (naturally) or in transfected cells, they might (1) traffic to the same place (e.g., the same cholesterol-enriched domains of the PM usually favored by GPI-anchored proteins), (2) interact directly, and/or (3) share protein partners. Both cell biological and biochemical approaches have been used to explore these interrelated concepts.

3.5.1 Cellular Locales

By Triton X-100 extraction and Nycodenz step-gradient fractionation, both PrP^C (in brain) and Dpl (in testis) were found in low-density, raft-associated fractions. However, using a combination Triton X-100 plus immunoprecipitation protocol, the proteins naturally present in testis extracts were not found to coimmunoprecipitate, suggesting they localize to different rafts [101]. Also, in MDCK cells, PrP^C and Dpl were sorted to different surfaces (basolateral and apical, respectively) [102]. These findings stand in contrast to three other studies, however. Using polarized

Fisher rat thyroid cells [103], Dpl and PrP^C were found to colocalize to the same basolateral surface, to be associated with rafts, and to be coimmunoprecipitated either with each other or each in association with the raft protein, flotillin. Two other studies used neuroblastoma cells. As reported by Massimino et al. [84], SK-N-BE cells transfected with either PrP- or Dpl-fluorescent fusion proteins exhibited a similar fluorescent signal distribution in the PM and Golgi apparatus and exhibited similar fractionation following Triton X-100 extraction and step-gradient fractionation. At the neuroanatomic level, a different type of commonality was observed in that fusion proteins composed of PrP or Dpl joined to an immunoglobulin constant region revealed similar binding to granule cells of the cerebellum [104], a result that was not interpreted in favor of direct binding to PrP^C, but in terms of binding to another type of molecule.

3.5.2 Direct Interactions of Dpl with PrP and Sho?

Antagonistic interactions between PrP^C and Sho vs Dpl or internally deleted PrP have been reported in genetic and cell biological paradigms, raising the question of direct physical contacts between these two proteins. This has now been considered by a number of groups, with several of them reporting interactions. Thus PrP/Dpl interactions were described for purified recombinant proteins in ELISA format and in co-immunoprecipitations, though in the latter context the possibility for tripartite interactions of the sort "PrP/shared protein/Dpl" cannot be excluded [103, 105, 106]. In addition, Watts et al. have demonstrated that immunoprecipitations with FLAG-tagged Dpl enrich the endogenous wt Sho present in N2a cells [106].

3.5.3 Dpl Partners and Interactomes

Beyond abrogation by PrP^C, it has been suggested that Dpl might induce neurodegeneration by interacting with the plasma metalloproteinase inhibitors rat α-1-inhibitor-3 and α-2-macroglobulin [105]. These protease inhibitors were identified using a Dpl fusion protein as bait to probe electrophoretically-fractionated proteins derived from dissected cerebellar tissue. Following in-gel digestion, the candidate partners were identified by mass spectroscopy. A hypothetical pathogenic pathway can be inferred involving disinhibition of surface metalloprotease activity, but this remains to be assessed in an in vivo context. Using two-hybrid screens, Dpl was reported to interact with the laminin receptor protein (LRP) and with the human receptor for activated C-kinase 1 (RACK1) [107, 108]. The possibilities of artifacts in expression of mammalian glycoproteins in the yeast cytoplasm notwithstanding, these findings do raise issues of neoplasia and signal transduction that are considered in Sect. 3.3. Lastly, a more global approach was adopted in N2a cells transfected with FLAG epitope-tagged Dpl and PrP (and Sho), formaldehyde cross-linking and immunoprecipitation followed by mass spectroscopic analysis of tryptic peptides revealing a number of common partner proteins [106]. These data led to

the conclusion that the two proteins occupy common membrane microdomains. The preponderance of cell-surface protein partners is compatible with direct immunocytochemical staining for Dpl ([19, 84]), and perhaps with the suggestion that Dpl expression can modulate cellular migration capability in astrocytoma-derived cells and in HeLa cells [109].

The studies above consider binding from the starting perspective of using full-length PrP and Dpl "input," but it may also be important to examine processing events acting upon mature forms of the glycoproteins; in other words, to consider the possible downstream consequences of Dpl and PrP binding to each other or to shared partners. In this context Dpl has been noted to impact the steady-state level of the PrP endoproteolytic fragment called C1 [110]. Other posttranslational effects may yet remain to be discovered.

3.6 Reproductive Phenotypes and the Dpl Protein

As we alluded to above, a body of literature documents the expression of Dpl in the male reproductive tract and the "big picture" has Dpl in a starring role in the biology of this tissue. Reassuringly – given the contrasting situation for PrP itself – this view is backed up by the results of genetic ablation of *Prnd*, which results in male infertility [111–114]. Sites of expression described in the male genital tract include Sertoli cells, seminiferous tubules within in developing spermatids, and the epididymis [19, 74, 83, 115, 116]. With regards to the end product of gametogenesis, ejaculated mature sperm, there are contrasting reports as to whether Dpl expression is always associated with the acrosome in different mammalian species [117–119]. Also, some divergence is also seen with regards to in vitro fertilization experiments to pinpoint the stages where Dpl might be crucial. Specifically, there has been a debate about gross morphological alterations (or lack thereof) in sperm from homozygous null mice, the ability of sperm to penetrate the *zona pellucida* of wt oocytes in the first place, or a reduced ability for fertilization resulting from abnormal acrosome reaction followed also by an inability of fertilized oocytes to develop further than the morula stage [114, 120]. Beyond invoking the usual caveat of distinctions in inbred strains (129/Ola vs C57BL6/CBA), it is difficult to fathom the origins of these divergent datasets, given the penetrant null alleles used by both groups. Nonetheless, Paisley et al. described one molecular "lead" for go-forward studies, this being an accumulation of DNA damage (as measured by two assays) in the sperm from the homozygous null male parents [114]. What might be the cause of this damage? Here the authors refer to studies of *Rcm*0 mice that overexpress Dpl protein having increased levels of molecular oxidation products [121]. This, however, is a provisional explanation and it leads to new issues. First, the precedent oxidative damage was reported in the brain, not in the male genital tract or sperm. Second, studies of TgDpl(10329) mice with more rapid onset of clinical disease than *Rcm*0 mice (at 375 ± 8 vs 611 ± 12 days [122]) and with higher levels of CNS Dpl expression – and, by implication, with a greater propensity to accumulate

oxidative damage – failed to reveal elevated levels of either α-nitrotyrosine or of α-carbonyl modification of brain proteins [93]. The term "oxidative damage" can be a generic catch-all explanation in the field of neurodegeneration and here there might be something of the same danger in drawing an incorrect conclusion – or at least an imperfectly supported conclusion – about the consequences of deficiency for the Dpl protein. Given the concrete endpoint measure, the complete block on fertility of male *Prnd*$^{0/0}$ mice, it might be time to reopen the books on this mechanistic question, starting perhaps with the new inventory of candidate protein partners [106] (see Sect. 3.5.3) and new ideas about modulation of membrane pores by ΔPrP [123, 124], which is structurally similar to Dpl.

3.7 CNS Neurodegeneration Associated with the Dpl Protein

It is now time to consider the neurotoxic potential of the Dpl protein, as already alluded to several times in the foregoing text. The neurodegenerative impact of ectopically expressing Dpl in the CNS was stumbled upon over 10 years ago and can now be appreciated as a potent and replicated biological phenomenon. While the origins of the original Dpl neurodegenerative "syndrome" derive ultimately from imprecise genetic engineering of the *Prnp* locus [125] such that residual 5′ *Prnp* promoter sequences drive the *Prnd* coding region [19], the phenomenon has been, and continues to be, studied by a number of laboratories. There are two likely reasons. First, the natural domain structure of wt Dpl has an uncanny similarity to neurotoxic internally deleted forms of PrP such as Δ32–121 or Δ32–134 [46], providing a convergence and verification of two independent research themes. Second, and probably more important, because Dpl's neurotoxic activity manifested in *Prnp*$^{0/0}$ mice can be counterbalanced by reintroduction of wt PrP^{C}. This in turn opens a window into PrP^{C} function and provides a workable "bioassay." Accordingly, over the intervening years the "push–pull" paradigm whereby Dpl causes neurotoxicity and PrP^{C} provides neuroprotection has been use in the following ways: (1) determining by deletion analysis the necessary determinants within PrP^{C} present in *trans*, (2) supplying active regions of PrP^{C} in *cis* by grafting them on as Dpl N-terminal extensions by use of fusion proteins, and (3) exploring the *cis*/*trans* effects in a cell biological context whereby Dpl or ΔPrP is expressed in one type of cell (by judicious selection of transgene promoters) and with wt PrP^{C} expressed in another type of cell. Here we can summarize a series of consensus observations from the sum total of such studies:

1. That neurotoxic activity is inextricably derived from synthesis of the Dpl protein and does not derive from some other subtlety/idiosyncrasy of coding or noncoding sequences within the *Prnp–Prnd* ("*Prn*") gene region of mouse; this is established definitively by use of Dpl transgenics with transgene arrays integrated at distal chromosomal loci and by the use of single vs double k/o mice with differing lengths of deletion interval [113, 122, 126].

2. The "toxic repertoire" of neuronally expressed Dpl is especially evident in the cerebellum and can include both Purkinje cells (as per the original Ngsk mice [125] and transgenics made with an "L7" gene promoter [126]) and granule cells (as per TgDpl transgenics made with the hamster PrP cos.Tet vector [122]). It is also noted that Dpl causes leukoencephalopathy in the absence of oligodendrocytic PrP^C [127] and that white matter alterations are a striking feature of Dpl expressed from an inducible Dpl transgene (J. Yang and DW, unpublished). One newer possibility is that the observed spectrum of toxic potential is shaped by region-specific maturation of Dpl, and thus Purkinje-cell degeneration in prion protein-deficient mice is associated with a cerebellum-specific Dpl protein species signature [128].
3. The mode of cell death induced by Dpl has frequently been equated with apoptosis [122] (see also Sect. 3.8), although autophagy has recently been invoked in addition [129]. There is controversy as to the involvement of Bax in modulating Dpl-initiated apoptotic pathways [130, 131].
4. PrP^C's protective activity can be titrated against Dpl expression level by alterations in endogenous *Prnp* copy-number or transgene array copy-number but the highest levels of Dpl can out-compete PrP^C-directed protective activity [122, 132, 133]. With generally similar results obtained with ΔPrP expression, Dpl and ΔPrP are assumed to modulate a shared, discrete biochemical pathway [126, 134].
5. PrP^C's protective activity, assessed in *trans* in genetic paradigms, maps to the N-terminus and can also be supplied in *cis*. For example, N-terminal residues 23–88 of PrP containing the unique octapeptide-repeat region are crucial for preventing Purkinje cell death in $Prnp^{0/0}$ mice expressing neuronal Dpl [135] while other studies used a PrP-1-124 determinant [136]. This conclusion is intuitively satisfying as the carboxy-terminal globular domains of wt PrP and wt Dpl were already known to have similar three-dimensional folds [86].
6. From a cell biological perspective, PrP^C expressed from a neuron-specific enolase promoter could rescue granule cell degeneration but not a Dpl-specified leukoencephalopathy. In a reverse fashion, PrP expressed in *trans* from a myelin basic protein promoter could rescue leukoencephalopathy but not granule cell death [127].

Beyond the suggested areas of consensus in points 1–6, other workers have gone further to describe late-onset olfactory deficits and mitral cell loss in Rikn $Prnp^{0/0}$ mice with ectopic expression of Dpl [137]. Additional avenues of investigation involve linking the push–pull effect into the realm of delineated signaling pathways. Thus expression level of type 1 inositol 1,4,5-trisphosphate receptor, a calcium-release channel protein, was found to be decreased in the Ngsk line of $Prnp^{0/0}$ mice. Reduced brain-derived neurotrophic factor (BDNF) and ionotropic glutamate receptor subtype 2 were also observed, along with reduced DNA binding by expression of nuclear factor of activated-T cells (NFAT). These effects could be rescued by reintroducing PrP^C expression [138]. Lastly, based upon the responses of Rikn $Prnp^{0/0}$ splenocytes to cell proliferation after LPS stimulation (vs the responses of $Prnp^{+/+}$ or Zrch I $Prnp^{0/0}$ control splenocytes), Kim et al.

have suggested that the push–pull concept of Dpl/PrP^C signaling may go beyond the degeneration events dictated by CNS cells and may involve a type of cross-talk with systemic events, a view emerging from the concept of "neuro-immunomodulation" [139].

Besides comments provided here, this field has been reviewed extensively and the reader is directed to other articles for a broader perspective [81, 111, 140, 141]. Lastly, before leaving the realm of "whole animal" models, it is important to underscore that the neuropathology encountered from neuronal expression of Dpl diverges from that seen in prion infections and genetic prionopathies. For example, there is no evidence that Dpl itself misfolds into aggregates detectable by light microscopy (see also Sect. 3.4).

3.8 Push–Pull Effects of Dpl and PrP^C Assessed in the Petri Dish

As can be appreciated from the foregoing text, signaling pathways that might emerge from the Dpl/PrP push–pull paradigm are not guaranteed to have relevance to human and livestock prion diseases but, instead, are more likely to open doors to PrP^C's somewhat mysterious mode of action. This notwithstanding, the molecular underpinnings of Dpl/PrP^C antagonism might be advantageously appraised in cell-culture systems. Here the ability to address autonomous and nonautonomous effects by coculture, to dose with agonists, to image in real-time, and to use tracers can all afford an opportunity to "drill down" to the relevant biochemical interactions and pathways. In fact, although there is one report to the contrary [142], data from several different paradigms suggest that robust antagonistic effects can indeed be observed in the Petri dish.

Onodera and coworkers scored an early achievement in this area by deriving a hippocampal cell-line HpL3–4 from the CNS of Dpl-expressing *Rikn Prnp*$^{0/0}$ mice [143]. When subjected to serum withdrawal, these cells undergo a wave of cell death with the DNA fragmentation hallmark of apoptosis. Although Dpl expression from chimeric transcripts in this cell-line was sufficiently low to require loading of 200-μg aliquots of protein for blot analysis, synthesis of the protein was confirmed [144]. In terms of mechanism, the effect of serum withdrawal was not to modulate the level of chimeric *Prnp*/*Prnd* transcripts. Delivery of "additional" Dpl by a vector, as well as commensurate rescue by PrP overexpression, confirmed that the Dpl/PrP push–pull paradigm could operate in these cells and, in addition, confirmed that the whole system can function in a cell autonomous manner. Building on this system, fusion of PrP N-terminal sequences (residues 1–124) abrogated the toxic effects of Dpl [145]. With regards to the question of whether the low level of Dpl present in the original, untransfected HpL3–4 cells *determined* cell death upon serum deprivation, a follow-up experiment examined the impact of serum withdrawal-induced apoptosis in cells made from ZrchI *Prnp*$^{0/0}$ mice (i.e., without Dpl overexpression). These experiments establish that the effects of serum

withdrawal and the converse effects of prion protein reconstitution scored in hippocampal cell lines are largely independent of Dpl [146] and that PrP^C protects hippocampal cells from deprivation of factors present within serum.

These data of Nishimura et al. dovetail with the larger literature on PrP^C's neuroprotective function and also reveal a parallel to titration effects perceived in transgenic (Tg) mice. Nonetheless, the intact hippocampal formation is not notably affected in TgDpl mice [122] whereas CGN are. Accordingly, recombinant Dpl protein (refolded to an α-helical form) was assessed on CGN neurons from *Prnp*$^{0/0}$ mice where it was found to be toxic. This form of toxicity involved stimulation of nitric oxide production via activation of the nitric oxide synthases nNOS and iNOS [147]. Partially analogous studies failed to register a protective, titrated effect of PrP genotype when using recombinant Dpl (which was, however, toxic to CGNs), but did when Dpl was delivered by transgenes. These studies thereby allowed deletion mapping of protective and toxic determinants in the PrP and Dpl [148]. Following the demonstration of Sho as an authentic CNS glycoprotein, these studies were extended to register a protective effect of wt Sho and an impact of the Sho hydrophobic tract in this process [4]. Interestingly, use of human SH-SY5Y neuroblastoma cells produced similar results for the effects of Sho [3]. Earlier studies with these cells had already described similar proapoptotic effects of Dpl and PrP Δ32–121 occurring with the same apoptotic process, likely with a triggering of a mitochondrial pathway contributing to the end result [149]. Moreover, using toxicity to SH-SY5Y cells as an endpoint for administration of recombinant Dpl, not only could rescue by PrP^C be demonstrated, but toxicity could be "mapped" to residues 81–122 in close accord with Drisaldi et al.'s genetic mapping of the toxicity determinant to residues 101–125 [148]. In different types of cells – astrocytes and N2a cells – Dpl was found to induce apoptosis in N2a cells and rat primary astrocytes via a caspase-10 mediated pathway that was counteracted by cellular PrP [150]. Lastly, rather than look at cytotoxicity, another paradigm has focused upon Ca^{2+} as an outcome measure. Using Ca^{2+}-sensitive aequorin tracers, the presence of PrP^C introduced by acute transfection increased Ca^{2+} in subplasma membrane domains of Chinese Hamster Ovary cells. PrP^C also limited Ca^{2+} release from the endoplasmic reticulum and Ca^{2+} uptake by mitochondria, a property that would dampen the triggering of cell death pathways. In contrast to PrP^C, cells expressing Dpl displayed opposite effects, which could nonetheless be normalized by coexpression of PrP^C [151].

The sum total of these cell paradigms is to demonstrate that the antagonistic effects of PrP^C and Dpl can be recreated in simple systems, often monocultures, and further underscore cytoprotection as a recurrent theme in PrP^C's repertoire.

4 Concluding Remarks: Two Views and a Secret

Aerial perspectives can give access to inaccessible terrain and a contemporary catchphrase is "the view from 60,000 feet" (the view from 18,288 m is rarely described). So, at this juncture, it is useful to consider a 2011 aerial perspective to

collate a larger view on PrP^C, Dpl, and Sho, the mammalian cellular prion gene family members that encode functional proteins.

4.1 Gain-of-Function

PrP is distinct in that it emerged from the study of diseases, with these occurring in no less than three varieties: that is to say, infectious, sporadic, and genetic. Indeed, it has been speculated that even if PrP was not discovered by protein purification from scrapie-infected brains in the 1980s, it would have inevitably been discovered by positional cloning of Gerstmann–Straussler–Scheinker mutations located on the short arm of chromosome 20 [152]. On the other hand, Dpl and Sho are not yet represented by "Doppel-opathies" or "Shadoo-opathies" and thus seem benign by comparison. Given the power of disease ascertainment in modern-day biomedical science, this division is likely real – but why? The likely explanation comes from consideration of whether the proteins encoded by *PRNP*, *PRND*, and *SPRN* can exhibit "gain-of-pathologic function." For PrP^C this is an open and shut case but for Dpl and Sho there is a paucity of data that they can refold to pathological forms. One possibility is that when *PRND* was generated by gene duplication of *PRNP*, its relegation to expression within the nether regions of mammalian anatomy may have placed it in a context where misfolding is more tolerated and merely results in some cell loss from within a renewable population. Also, as noted above, the extra disulfide linkage in Dpl may greatly stabilize the protein. Sho contains a central hydrophobic region that is loosely analogous to PrP sequences that are refolded in PrP^{Sc} and it is expressed in the CNS, yet an entity that we might term as "Sho^{Sc}" has yet to be found in an in vivo situation. We now know that Sho can be refolded to an amyloid-like form in vitro [153] but the in vivo correlate of this might conceivably be a "physiological amyloid" or might never be allowed to occur in the first place. Thus, unlike PrP^C (or Dpl), Sho does not have the benefit of globular C-terminus and, relative to PrP^C, seems to possess greater sensitivity to proteolytic digestion and more rapid metabolic turnover [153].

4.2 Loss-of-Function

This comprises another route to disease outcomes and pathogenesis and here the decks are not stacked neatly into PrP vs Dpl/Sho camps. To the best of our knowledge, inactivating mutations have never been described for *PRNP*, nor is there copy-number variation, and even stop codons in the middle of the ORF are pathogenic by a "gained" propensity for amyloid formation [154]. Given PrP's troublesome nature, this seems surprising but the origins for this observation might comprise an almost indispensible (but obscure) normal function, coupled with a propensity for average disease onset subsequent to the reproductive years.

Dpl appears quite different. Although not featured in the "morbid map" in Online Mendelian Inheritance in Man (OMIM: http://www.ncbi.nlm.nih.gov/Omim), the phenotypic prediction for genetic deficiency is clear and corresponds to male sterility, something perhaps unlikely to turn up in a neurologist's office. Sho is different yet again with a "first" for the prion gene superfamily – natural null alleles. These were described in two apparently independent families in the UK in the form of frameshifts positioned approximately one third of the way into the ORF [39]. The mutations were discovered as a result of scrutiny of a series of sCJD and vCJD patients, occurring in the latter but not, to date, in normal controls. These unusual alleles were present in heterozygous form and imply a potentially causal role in vCJD susceptibility. Since Sho expression is limited almost exclusively to nervous system tissue, a "trivial" mechanism of action such as control of ingress of vCJD prions across the digestive tract seems unlikely. The alternative is a type of active role in prion replication or neuropathogenesis. Returning to the theme of loss-of-function outside of prion infections, the question arises as to what would be the phenotypic outcome in the case of consanguinity in the *SPRN* frameshift kindreds (i.e., leading to homozygous null $SPRN^{0/0}$ offspring). There is an imperfect precedent from horses, who are beloved by many in spite of their lack of an *SPRN* gene [6], so the answer here may be that Sho is "PrP-like" (as per $Prnp^{0/0}$ mice) in that there is no overt phenotype. This consideration brings us back full circle to the last problem and to a "secret" – namely the elusive physiological functions of PrP^C and the PrP supergene family.

4.3 Entering a new molecular ecosystem?

An almost unique place within protein databases and twenty-five years of study has underscored the still enigmatic role of PrP^C in the neurobiology of healthy organisms. With a possible origin from the ectodomain of Zip metal transporters, it seems that PrP has evolved to a modern-day function that does not have a precedent amongst cell-surface proteins, perhaps representing a new type of plasma membrane ecosystem. In a context where we await a clarifying insights to unify a panoply of PrP^C data into a simple mechanistic mode of action, the GPI-anchored N-glycosylated Doppel and Sho proteins offer parallel avenues for study. With one or both proteins related to PrP by gene duplication events, mammalian forms of Sho and Dpl can be equated with the front and back halves of full-length PrP. These other proteins may be simpler molecular entities that can be pursued to advantage in functional studies. Dpl has a similar 3D "fold" to PrP^C and a profound role in successful gametogenesis that calls for close scrutiny. A case for deeper study can also be made for Sho, a CNS expressed glycoprotein with many overlaps to defined elements of PrP biochemistry and, possibly, a common role in embryogenesis. In an aerial view of biomedical research, Dpl and Sho might be considered as adjacent 'islands in the sky' in a PrP archipelago. The coming years of exploration should be extremely fruitful.

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Fungal Prions: Structure, Function and Propagation

Mick F. Tuite, Ricardo Marchante, and Vitaly Kushnirov

Abstract Prions are not uniquely associated with rare fatal neurodegenerative diseases in the animal kingdom; prions are also found in fungi and in particular the yeast *Saccharomyces cerevisiae*. As with animal prions, fungal prions are proteins able to exist in one or more self-propagating alternative conformations, but show little primary sequence relationship with the mammalian prion protein PrP. Rather, fungal prions represent a relatively diverse collection of proteins that participate in key cellular processes such as transcription and translation. Upon switching to their prion form, these proteins can generate stable, sometimes beneficial, changes in the host cell phenotype. Much has already been learnt about prion structure, and propagation and de novo generation of the prion state through studies in yeast and these findings are reviewed here.

Keywords Molecular chaperone · *Podospora anserina* · Prion · Prion propagation · Propagons · Yeast (*Saccharomyces cerevisiae*)

Contents

M.F. Tuite (✉) and R. Marchante
Kent Fungal Group, School of Biosciences, University of Kent, Canterbury, Kent CT2 7NJ, UK
e-mail: M.F.Tuite@kent.ac.uk

V. Kushnirov
Russian Cardiology Scientific Industrial Complex, Moscow, Russia

1 The Discovery of Yeast Prions

1.1 The [PSI$^+$] *and* [URE3] *Determinants*

The realisation in 1994 that two unusual genetic traits in the budding yeast *Saccharomyces cerevisiae* are prion-mediated [1] provided a highly tractable platform to explore the behaviour and impact of these novel, protein-only infectious entities. The two heritable traits in question, [*PSI*$^+$] and [*URE3*], emerged from classical genetic screens [2, 3] and shared an unusual "non-Mendelian" pattern of inheritance in genetic crosses. When a [*PSI*$^+$] or [*URE3*] mutant was back-crossed to a wild type strain, the trait was dominant but failed to segregate in meiosis as a single gene trait. In other words, all four products of meiosis (in the case of yeast these are haploid spores) inherited the underlying "genetic" determinant and expressed the associated phenotype. That both traits can be transmitted from cell-to-cell in the absence of nuclear fusion (i.e. by cytoduction) confirmed that the underlying genetic determinants must be located in the cytoplasm. The failure to eliminate the traits by growth of cells in low concentrations of ethidium bromide ruled out the possibility that they were mitochondrial genes, nor could they be linked to any other nucleic acid species known to reside in the yeast cytoplasm [4, 5]. Their physical basis thus remained a puzzle and, although a wide range of scenarios were rehearsed prior to 1994 (see for example [6]), the possibility that they were protein-only "genetic determinants" was not given serious consideration until the paradigm changing study of Wickner [1].

Some quarter of a century after their discovery and against the backdrop of the increasing acceptance of Stanley Prusiner's prion hypothesis [7], it finally emerged that both the [*PSI*$^+$] and [*URE3*] traits were caused by prions. In considering the molecular nature of the [*PSI*$^+$] and [*URE3*] determinants Wickner [1] had used several genetic criteria to confirm their prion-like nature and to identify the corresponding prion proteins as Sup35 and Ure2 respectively. These criteria included the ability of the determinant to arise de novo at a frequency that was significantly elevated by overexpression of the corresponding prion protein [1, 8, 9] and then showing that the determinant could be reversibly eliminated by chemical agents, specifically guanidine hydrochloride [1, 10, 11]. Both [*PSI*$^+$] and [*URE3*]

satisfied these criteria and this, coupled with the requirement for the corresponding protein to be expressed in the cells for continued propagation of the trait, pointed strongly at a prion-like mechanism.

In taking up the prion form, the corresponding yeast protein was assumed to be either non-functional or at least had reduced function that led to the phenotype associated with the presence of the prion. In the case of [*PSI*$^+$], the loss of function of Sup35 (a translation termination factor eRF3 [12]) resulted in an increase in tRNA-mediated nonsense suppression [2] while loss of function of Ure2 in [*URE3*] cells led to a modification in the availability of certain nitrogen sources [3]. Subsequent to Wickner's original study [1] it was shown that in [*PSI*$^+$] and [*URE3*] cells the Sup35 and Ure2 prion proteins respectively were largely present in a high molecular weight form [13–15] that could catalyse the in vitro aggregation of the soluble form of that protein into aggregates [16–18]. That the two proteins were able to switch from a soluble form to an aggregated form in [*PSI*$^+$] and [*URE3*] cells was further demonstrated by fusing the corresponding proteins to green fluorescent protein (GFP) and showing they formed discrete cytoplasmic aggregates (often referred to as foci) but only if the cells already carried the respective prion determinant [14, 16].

The final proof that the [*PSI*$^+$] and [*URE3*] were prions came from the demonstration that aggregated forms of the corresponding proteins, either generated in vitro or isolated from [*PRION*$^+$] cells, could be used to "infect" prion-free [*prion*$^-$] cells and induce the formation of the respective determinant that was then stably inherited in cells otherwise lacking the determinant [19–21]. Furthermore, that the in vitro form of these proteins was amyloid in nature, confirmed an earlier suggestion [22] that, like their mammalian counterpart, yeast prions, are examples of transmissible amyloids.

1.2 The Podospora anserina *[Het-s] Prion*

While the pioneering studies on fungal prions concentrated on the [*PSI*$^+$] and, to a lesser extent, the [*URE3*] determinants, it soon emerged that a second fungus contained a non-Mendelian genetic trait that was also associated with a prion, namely the [Het-s] trait of the filamentous ascomycete *Podospora anserina* [23]. This trait was first identified as the genetic basis of vegetative incompatibility, a process that prevents the formation of mixed multinucleate heterokaryons between two different strains of the fungus. The death of the heterokaryon prevents the transmission between strains of potentially deleterious viruses and other cytoplasmic nucleic acids. Using the same approaches as those applied to the [*PSI*$^+$] and [*URE3*] yeast prions, the underlying prion protein was identified as the HET-s protein. In the [Het-s] strains the HET-s protein was present in an aggregated form [24] and these protein aggregates were able to induce the appearance of the [Het-s] prion when introduced into [Het-s*] cells that only had the soluble form of the protein [25].

Since the original reports of fungal prions in *S. cerevisiae* and *P. anserina*, the number of confirmed prions in *S. cerevisiae* – as discussed below – has now risen to 8 with another 20 or more potential prions awaiting further investigation and validation [26, 27]. Although a number of these functionally unrelated prion proteins share common sequence features, they show little overall amino acid sequence similarity.

Studies on fungal prions have largely centred on the [PSI^+] prion and its prion protein Sup35 and have addressed – and in many cases answered – the most important questions in prion biology, in particular:

- What is the relationship between protein conformational changes and prion transmission/infectivity?
- What is the mechanism of propagation of the prion form and what role(s) do cellular factors play in this – if any?
- How do prions arise de novo?
- What impact do prions have on the host cell biology and evolution?

In this chapter we will describe the current status of these studies and show how they have informed research on the mammalian PrP prion protein.

2 Identifying Fungal Prions

The [PSI^+] and [*URE3*] prions were discovered by serendipity rather than emerging from a rational screen for prions. Subsequently new yeast prions have been, and continue to be, uncovered through a more targeted search strategy. This rational approach was triggered by the early realisation that yeast prion proteins have a common overall architecture [26, 28, 29]. The architectural features are a functional domain that mediates the cellular function of the protein coupled to a region located at either the N- or C-terminus of the protein that, although not essential for protein function, is essential for the protein to be able to switch to a stable and heritable prion conformation. Consequently these regions are termed prion-forming domains (PrD) and are a highly flexible region of the protein molecule that is rich in Gln and Asn residues [30, 31]. Yet, of the 200 or so proteins in yeast with this architecture, only 29 pass additional functional tests for prion behaviour [26] (Table 1) and of these only 8 have so far been fully validated as prions in a natural context, i.e. the native full-length protein is able to form a heritable and infectious altered conformer in the yeast cell. Two further proteins have been designated as prion forming, but do not yet satisfy all of the criteria applied by Alberti et al. [26]; they are Mca1p, a cysteine protease similar to mammalian caspases that forms the [MCA^+] prion [32, 33] and the transcriptional activator Sfp1p which can generate an atypical prion called [ISP^+] [34].

Table 1 Prions and putative prion proteins in the yeast *Saccharomyces cerevisiae*

Cellular function[a]	Confirmed prions	Putative prion proteins[b]
Transcription		
Regulation	Cyc8/[*OCT*$^+$], Mot3/[*MOT3*$^+$] Ure2/[*URE3*$^+$], Sfp1/[*ISP*$^+$]	Gln3
Mechanism		Gts1, Pgd1, Rlm1, Pdr1
Chromatin	Swi1/[*SWI*$^+$]	Sap30
Translation		
Mechanism	Sup35/[*PSI*$^+$]	Mrn1
Ribosome biogenesis		New1, Nrp1
mRNA biology		
mRNA processing[c]		Puf2, Lsm4, Pub1,Hrp1, Ngr1, (Rbs1)
mRNA export		Asm4, Nsp1
Protein modification		Cbk1, Ksp1
Other	Mca1/[*MCA*$^+$]	Gpr1
Unknown	Rnq1/[*PIN*$^+$]	YBR016w, YBR022c, YBL081w

[a]Based on information available in the *Saccharomyces Genome Database* (www.yeastgenome.org)
[b]As defined by the following assays: form aggregates when fused to GFP, form SDS-resistant protein aggregates in vivo, form amyloids in vitro, impart prion-like behaviour on Sup35 C-domain [26]
[c]Includes mRNA splicing, polyadenylation and turnover

3 Prion Proteins and Their Cellular Roles

That an alternative conformational form of the mammalian PrP protein (PrP^{Sc}) is a major component of the infectious entity that causes the TSEs is well established and widely accepted [35]. Yet, remarkably, we know little about the cellular function of PrP in its soluble non-prion form (PrP^{c}) or indeed why it causes neurotoxicity when it switches to its infectious PrP^{Sc} prion form. PrP may have a pleiotropic role in vivo that may reflect an activity in one or more cell signalling pathways [36]. In yeast, with the number of verified and potential fungal prions now approaching 30 (Table 1), it is immediately evident that a wide variety of proteins that fall into different functional classes can form prions in this "simple" unicellular eukaryote. For example, if one just considers the two original fungal prions, [*PSI*$^+$] and [*URE3*]; in one case (Sup35/[*PSI*$^+$]) the protein plays a global role in mRNA translation, while in the other (Ure2/[*URE3*]) the protein acts as a co-repressor of the transcription of a subset of unlinked genes. The ability to switch to an altered functional state via a prion-like mechanism is thus not restricted to any one class of protein or to a specific sub-cellular compartment.

3.1 Prions as Global Gene Regulators

While not all the functions of the verified and putative yeast prions have been established, nevertheless it is already evident that fungal prion proteins are involved in the functioning and/or regulation of several different cellular processes. In particular, a number play important roles in gene regulation, either at the

transcriptional or post-transcriptional level, and as such have the potential to modify gene expression on a genome-wide scale [27]. In addition, two fungal prions, [Het-s] in *P. anserina* and [MCA^{+}] in *S. cerevisiae* may play a role in the control of programmed cell death.

3.1.1 Transcriptional Regulation by Prions

Five of the verified yeast prion-forming proteins, Ure2, Mot3p, Cyc8, Swi1 and Sfp1, are transcriptional regulators either acting as activators or repressors. In switching to their prion form, the normal role of the protein is over-ridden and this can lead to changes in the expression of a number of co-regulated genes. For example, in its non-prion state (i.e. in [*ure3-o*] cells), Ure2 functions as a co-repressor by binding to two transcriptional activators (Gln3 and Gat1) of genes that are regulated by nitrogen catabolite repression [37, 38] (Fig. 1). By so doing, Ure2 prevents these two transcription factors from entering the nucleus and activating target genes such as *DAL5*, which encodes a permease required for the uptake of poor nitrogen sources such as ureidosuccinate (USA). When Ure2

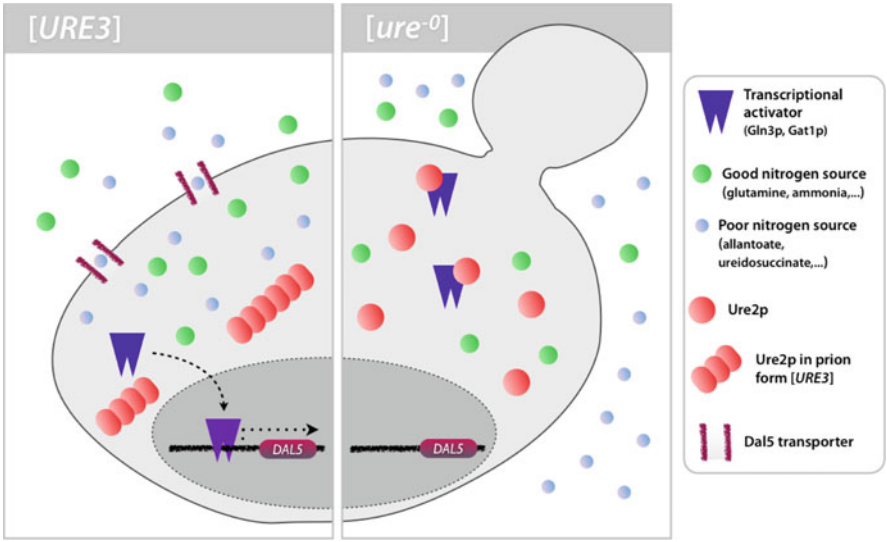

Fig. 1 Nitrogen uptake regulation by the [URE3] prion in *Saccharomyces cerevisiae*. In prion-free [ure^{0}] cells (*right panel*), Ure2 is soluble and can bind the transcriptional activators Gln3 and Gat1. This binding is dependent on the presence of good nitrogen sources such as glutamine or ammonia. In prion-containing [*URE3*] cells (*left panel*) Ure2 is aggregated and unable to bind Gln3/Gat1, resulting in the translocation of these transcriptional activators into the nucleus. This in turn results in the activation of transcription of a series of genes including *DAL5* which encodes a membrane transporter required for poor nitrogen sources such as allantoate and succinate. Consequently, poor nitrogen sources can be taken up by [*URE3*] cells even in the presence of rich nitrogen sources

switches to its prion form (i.e. in [*URE3*] cells) the interaction with Gln3/Gat1p is blocked and they are now able to cross the nuclear membrane and carry out their transcriptional role. This "loss-of-function" phenotype is identical to that seen when the *URE2* gene is inactivated by mutation [1].

The Swi1 [39] and Cyc8 [40] prion proteins potentially have a more global impact on gene transcription as both proteins regulate large gene networks with up to 400 genes. Certainly cells carrying either the [SWI^+]/Swi1 or [OCT^+]/Cyc8 prions show a range of phenotypes distinct from the [swi^-]/[oct^-] strains that are consistent with the prion impacting on global gene expression; for example strains carrying the [OCT^+] prion show defects in mating and sporulation and constitutive invertase activity [40]. A third prion formed by a transcription factor is the [$MOT3^+$] [26] and the Mot3 protein forms a co-repressor complex with a second protein (Rox1) that represses a range of genes including a number of anaerobic genes and genes involved in ergosterol biosynthesis.

3.1.2 Translational Regulation by Prions

At least one yeast prion can affect global mRNA translation, namely the [PSI^+] prion. As first described by Cox [2], the diagnostic phenotype of [PSI^+] cells is nonsense suppression, a phenotype that is a read out of the failure of the cell to recognise efficiently a termination codon (UAA, UAG or UGA) as a stop signal. When the stop codon has arisen by mutation within an open reading frame, [PSI^+]-mediated nonsense suppression can result in the cell being able to synthesise a certain level of a functional polypeptide from that open reading frame, thereby "suppressing" the mutant phenotype. This is best exemplified by the suppression of nonsense mutations in the *ADE1* or *ADE2* genes (Fig. 2a). The discovery that Sup35 (also called eRF3), the protein that forms the [PSI^+] prion, is a translation termination factor immediately provided an explanation for the molecular basis of the [PSI^+] phenotype.

Sup35 is a GTP binding protein that interacts with Sup45 (eRF1) to form the release factor that recognises an in-frame stop codon in an mRNA [12, 42]. The release factor triggers the discharge of the completed polypeptide chain from the ribosome. In a [PSI^+] cell the bulk of the Sup35 molecules are associated with high molecular weight SDS-resistant aggregates (Fig. 2b,c) and hence the efficiency of termination at stop codons is significantly reduced. This aggregation can be directly visualised in the living cell by fusing Sup35 to GFP [14] (Fig. 2d). The net outcome of this inactivation by aggregation is that the competition between near cognate tRNAs and the release factor for the stop codon shifts in favour of the tRNA, resulting in the stop codon being read as a sense codon up to 20% of the time.

[PSI^+]-mediated suppression of stop codons should result in C-terminal extensions to polypeptides encoded by wild type open reading frames. Such events on a global scale would be expected to be detrimental to the host given that the modified protein may be inactive and/or more susceptible to degradation. Yet there is no evidence that the presence of the [PSI^+] prion negatively impacts on cell growth

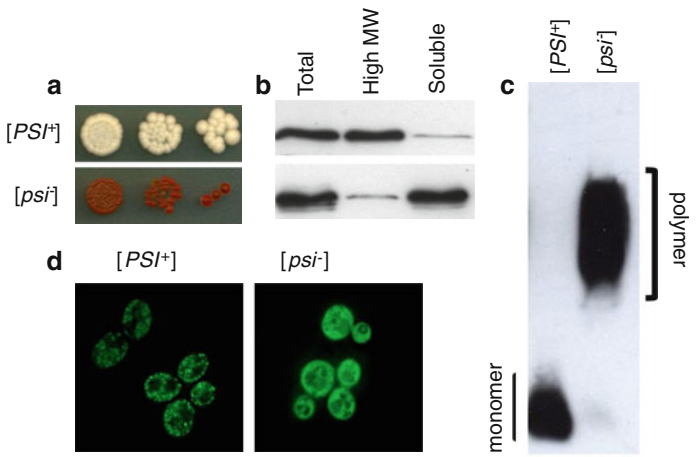

Fig. 2 The behaviour of the Sup35 (eRF3) protein in [PSI$^+$] and [psi$^-$] cells. (**a**) The presence of the [*PSI*$^+$] prion can be verified using a nonsense suppression assay based on the *ade1-14* allele. In [*psi*$^-$] cells the nonsense mutation is expressed leading to red pigment formation in yeast colonies. In the [*PSI*$^+$] strain the phenotype is suppressed giving normal colony colour. (**b**) In [*PSI*$^+$] cells Sup35 forms high molecular weight (HMW) aggregates that can be identified using a combination of high speed centrifugation and Western blotting for Sup35. Sup35 is only found in the soluble fraction in [*psi*$^-$] cells. (**c**) The aggregates formed by Sup35 in [*PSI*$^+$] cells are resistant to SDS and can be identified by SDD-AGE [41]. (**d**) When Sup35 is expressed as a fusion with green fluorescent protein (GFP) it form small aggregates detectable as fluorescent foci in [*PSI*$^+$] cells but not in [*psi*$^-$] cells

when compared to a prion-free [*psi*$^-$] cell [43]. In fact the presence of the [*PSI*$^+$] prion may be of benefit to the host cell under certain environmental conditions [44–46] indicating that, in contrast to expectation, termination readthrough has the potential to confer novel beneficial effects on the host. The only case to date where this has been directly demonstrated is for the *PDE2* gene which encodes a high-affinity cyclic AMP phosphodiesterase. In [*PSI*$^+$] cells the C-terminally extended protein has a higher intrinsic rate of turnover than the wild type form, resulting in a significant change in the levels of cyclic AMP [47] that may in turn impact on cAMP levels and potentially impacts on a range of cellular processes.

A second [*PSI*$^+$]-mediated translational event has a similar global effect on polyamine synthesis. The reduced termination efficiency seen in [*PSI*$^+$] cells results in an increase in a ribosomal frameshift (+1) during translation of the *OAZ1* gene which encodes antizyme, a negative regulator of polyamine biosynthesis. *OAZ1* consists of two overlapping out-of-frame ORFs that can be generated as an in-frame ORF via a +1 frameshift [48]. In [*PSI*$^+$] cells the elevation in frequency of frameshifting at this site leads to increased levels of antizyme which in turn reduces polyamine levels in the cell [49]. Reducing polyamine levels may impact on a range of cellular processes and may account for some of the other phenotypic differences reported between [*PSI*$^+$] and [*psi*$^-$] cells [44–46, 49].

3.2 *The* [PIN$^+$] *Prion*

[*PIN*$^+$] (originally designated [*RNQ*$^+$]) is the prion form of the glutamine and asparagine rich protein Rnq1, a non-essential protein of unknown cellular function. [*PIN*$^+$] was first identified as a cytoplasmic factor required for efficient de novo induction of several yeast prions, including [*PSI*$^+$] from whence it derived its name: ***P***SI ***IN***duction [29, 50, 51]. Unlike other yeast prions though, Rnq1 aggregation has not been associated with the loss of a cellular function, but rather with a gain of function; the ability to facilitate the de novo appearance of other prions. Although several different Asn/Gln-rich proteins can provide [*PIN*$^+$] function when over expressed [50, 52], Rnq1 is typically associated with [*PIN*$^+$] in most laboratory strains. A formal demonstration of the "protein-only" nature of the [*PIN*$^+$] determinant came from showing that infection of [*pin*$^-$] cells with in vitro-generated aggregates of the Rnq1 prion domain (which is located between residues 132 and 405) generated [*PIN*$^+$] cells [53].

The mechanism by which [*PIN*$^+$] aggregates promote prion formation has not been fully elucidated, but is either through acting as an initial imperfect template for Sup35 polymerization [50] or acts by titrating out undefined aggregation inhibitors [52]. The former model has received some direct experimental support. For example, the presence of [*PIN*$^+$] aggregates can shorten the lag period observed when Sup35 is allowed to self-polymerize in vitro [54] while Rnq1 and Sup35 prion domains can interchangeably cross-seed the in vitro polymerization of the other [55]. There is also evidence from in vivo studies of a Sup35:Rnq1 interaction [56]. These findings strongly support the existence of a physical interaction between Rnq1 and Sup35 during prion protein polymerization. However, the [*PIN*$^+$] prion is not required for the continued propagation of the [*PSI*$^+$] prion [57].

3.3 *Lethal Fungal Prions and Toxic Amyloids*

Fungal prions are, by-and-large, tolerated by the host whereas infection by a mammalian prion leads to the eventual death of the host. However, there are several examples in the fungal prion world where inheritance or de novo formation of a prion can lead to host death or impact on programmed cell death. The best understood example of prion-mediated cell death in fungi is that associated with the [Het-s] prion in *P. anserina* (Fig. 3). When the somatic cells of two genetically distinct strains of the filamentous fungus *P. anserina* meet, they fuse to form a multinucleate heterokaryon. Depending on the genotype of the two strains, the resulting heterokaryon may die, thereby preventing transfer of any potentially detrimental cytoplasmic components from one strain to the other [23, 24]. This form of innate immunity is called vegetative incompatibility (VI) and is controlled by nine different *het* genes one of which (*het-s*) encodes HET-s, a protein that can switch to the alternative conformational form that is the [Het-s] prion.

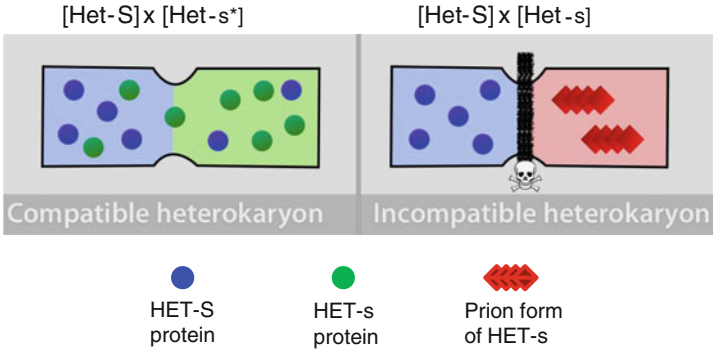

Fig. 3 [Het-s] prion–regulated heterokaryon incompatibility in *Podospora anserina*. The fungal hyphae expressing the HET-S protein (designated [Het-S]) are only able to fuse with hyphae expressing the soluble form of the HET-s protein (designated [Het-s*]) if HET-s is in the non-prion form of this protein. If the hyphae do express the prion form of HET-s (designated [Het-s]) then hyphal fusion triggers a lethal reaction at the point of contact, thereby preventing mixing of the cytoplasmic components of the two hyphal types

The [Het-s] prion was first identified over 50 years ago as a cytoplasmic genetic element controlling VI in *P. anserina* [58] but it was not until 1997 that [Het-s] was shown to be the prion form of the HET-s protein [23, 24]. Various strains of *P. anserina* carry the *het-s* allele referred to as *het-S* (the "grande S allele") that encodes a form of the protein (called HET-S) but is unable to switch to the prion form. VI is evident when a *het-s* strain carrying the [Het-s] prion fuses with a *het-S* strain. If the *het-s* strain lacks the [Het-s] prion (i.e. is [Het-s*]), then the cells fuse to form viable heterokaryons. The HET-S protein has 13 differences at the amino acid level compared to the HET-s protein although only one or two of these substitutions are necessary to differentiate the two in their behaviour in VI [59].

VI-induced cell death is accompanied by a variety of cellular events including a stimulation of autophagy and a global shut down in gene expression, but the precise mechanism of the resulting programmed cell death remains to be established [60]. The ability of the yeast metacaspase protein Mca1 – to form the prion [MCA^{+}] [32, 33] – might also lead to a change in programmed cell death in *S. cerevisiae* although the expected (but not yet proven) loss of Mca1 function would be expected to decelerate rather than accelerate programmed cell death [61].

While the presence of a fungal prion is well tolerated by the host cell under normal laboratory conditions, the presence of two prions, [PSI^{+}] and [PIN^{+}], can lead to cell death when levels of the corresponding protein (Sup35 and Rnq1 respectively) are elevated. In the case of [PSI^{+}]/Sup35, the resulting cell lethality is due to sequestration of Sup45 (eRF1), a protein that binds to Sup35 to give to rise to the essential translation termination factor activity [62, 63]. The overexpression of Rnq1 in a [PIN^{+}] strain leads to cell death most likely through the formation of a toxic oligomeric – possibly amyloid – form of the protein [64].

3.4 Other Prion-Like Elements in Fungi

Aside from the verified examples of phenotypes in yeast linked to prions, a significant number of other traits in fungi appear to be under the control of "atypical" genetic determinants. In those cases studied, a protein-based epigenetic element directs the inheritance of a novel trait, but, unlike the prion mechanism, the nature of the self-perpetuating change is not necessarily conformation based. For example, in the fission yeast *Schizosaccharomyces pombe,* the [Cin] epigenetic factor allows cells to survive in the absence of calnexin, an ER chaperone that is normally essential for cell viability [65, 66]. Although the [Cin] state is induced by the overexpression of the nucleolar protein Cif1 (calnexin-independence factor), the continued propagation of the [Cin] state does not require Cif1; thus, the physical nature of the [Cin] determinant remains to be established.

Two non-amyloid, but protein-based, self-perpetuating determinants have also been identified in *S. cerevisiae*. In one case, the vacuolar protease B (PrB) is produced, initially as an inactive precursor, and is processed to the active form by both mature PrB and by a second protease, protease A (PrA). In cells lacking PrA, PrB maturation depends exclusively on processed forms of itself, and hence a self-sustaining state designated [β] is established [67]. The second case is the [GAR^+] "prion" whose associated phenotype is resistance to glucosamine, a glucose mimetic that cannot be metabolised by yeast [68]. As with the "classical" prion determinants such as [PSI^+], the [GAR^+] determinant also shows a non-Mendelian pattern of inheritance, but rather than reflecting a conformational switch of a single protein to a prion form, the [GAR^+] "prion state" appears to arise as a consequence of a complex formed between two proteins, Pma1, a major plasma membrane pump, and Std1, which is involved in glucose signalling [68]. This two component complex trait may represent an inherited alteration in a regulatory pathway that gives rise to a stable self-perpetuating "autoregulatory state" [69]. Such a self-perpetuating "autoregulatory state" has also been suggested to mediate the white/opaque switch in *Candida albicans* [70]. Such behaviour could manifest itself as a "protein-only" epigenetic mechanism, but one that differs from the "classical" prion mechanism.

4 Propagation of the [$PRION^+$] Form

Prion proteins would be indistinguishable from other amyloid proteins if it were not for their ability to self-replicate and be transmitted to other cells, i.e. to propagate the [$PRION^+$] prion form. In yeast, continued propagation of the [$PRION^+$] state depends absolutely on at least two cytoplasmic chaperone proteins. Furthermore, the [$PRION^+$] form can be transmitted to daughter cells during cell division and, in so doing, transmits the associated phenotype to the daughter cell. Once established, the [$PRION^+$] form is relatively stable and can be propagated through many

hundreds of generations without loss. [*prion*$^-$] cells do arise de novo, but at a frequency of between 10^{-4} and 10^{-6} depending on the prion and/or the variant of that prion, a figure that is in line with inherited mutational changes in DNA sequence or chromosome loss. Fungal prions therefore represent epigenetic determinants of phenotype that are equally as stable as nuclear-encoded genetic traits.

Apart from the essential role of molecular chaperones (see below) the ability of fungal prions to form and be transmitted to daughter cells is dependent on their PrD and its primary amino acid sequence. These regions contain amino acids that bias them towards aggregation and may influence the range of structures that the prion protein can adopt. PrDs are thought to contain specific binding sites for the molecular chaperones which aid the shearing of prion aggregates into transmissible aggregates, termed propagons [22, 71]. Furthermore, the degree of similarity between primary amino acid sequences of prion proteins of different yeast species dictates the ability of the prion form to be transmitted between the different species, a so-called prion "species barrier" [72–74]. This barrier arises when a prion from one species is unable to cross seed the orthologous protein in a second yeast species and shows many parallels at the molecular and phenomenological level with the inability of mammalian PrP^{Sc} from one species being able to infect another species [75].

4.1 Generating Transmissible Amyloids: The Prion "Replication" Cycle

Early studies on [*PSI*$^+$] revealed that a key cellular factor required for [*PSI*$^+$] propagation is the molecular chaperone Hsp104 [76]. A yeast strain lacking the *HSP104* gene (i.e. *hsp104Δ*), although viable, fails to propagate any of the confirmed yeast prions and, in the case of [*PSI*$^+$], its overproduction also leads to elimination of the prion [76]. To explain this paradox, it was originally proposed that Hsp104 breaks amyloid fibres into smaller fragments [15, 22, 76], the same way as it breaks large aggregates of denatured protein [77].

Fragmentation of the prion protein aggregates ensures propagation of the [*PRION*$^+$] state in rapidly dividing cells in two ways. First, this ensures that there is a sufficient number of prion particles (i.e. propagons) to transmit to the daughter cells, thereby ensuring stable inheritance at cell division. Second, fragmentation accelerates the conversion of the [*prion*$^-$] protein monomers to the [*PRION*$^+$] form, since the conversion occurs only at polymer ends and fragmentation creates new polymer ends. When Hsp104 is overproduced, the current assumption is that it breaks polymers with increased efficiency and this leads to their complete dissolution and the regeneration of the non-transmissible [*prion*$^-$] form [22, 78, 79]. It may be considered that fragmentation completes the prion replication cycle (Fig. 4) whereas the other part of this cycle represents growth of the amyloid by seeded polymerisation, which does not require, at least in vitro, the help of molecular chaperones or additional cellular factors [17, 80].

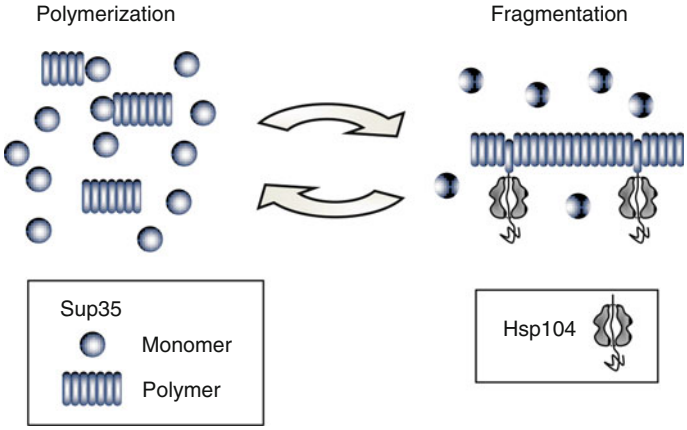

Fig. 4 Yeast prion replication cycle. Prion fibres grow by non-covalent polymerization of prionogenic proteins. Polymer fragmentation by Hsp104 completes replication cycle

One novel approach to studying the role of Hsp104 in vivo is to use guanidine hydrochloride (GdnHCl) as a reversible inhibitor of the ATPase activity of this chaperone [81–83], an activity crucial to its disaggregation function [77]. For example, this allowed the polymer fragmenting action of Hsp104 to be demonstrated in vivo by showing that GdnHCl leads to an increase in growth of Sup35 polymers with the rate of almost twofold per cell generation [41, 84]. Such a rate of growth is only possible if polymers are not being fragmented, i.e. the number of polymer ends remains unchanged. The remaining polymers continue to seed the polymerisation of newly synthesised molecules of Sup35, whose numbers approximately double every generation. As predicted by this mechanism, the levels of soluble Sup35 increased in GdnHCl-treated cells in inverse proportion to the decreasing number of prion polymers per cell. The direct visualisation of the impact of inhibition of Hsp104 activity by GdnHCl on the remodelling of Sup35-GFP aggregates in a [PSI^+] strain further supports the idea that the loss of chaperone-mediated fragmentation leads to an increase in polymer size and subsequent retention by the mother cell [85, 86].

The polymer fragmenting action of Hsp104 can be recapitulated in an in vitro system composed from purified Sup35 and high levels of Hsp104 [78], although other studies have failed to demonstrate this, e.g. [87]. One explanation for the discrepancy might be the requirement for additional chaperones at normal physiological levels to facilitate efficient fragmentation of the polymers. Recent studies have pointed towards an essential role for Sis1, a chaperone belonging to the Hsp40 class. Because Sis1 is essential for viability, the nature of propagation defects cannot be confirmed directly in a *sis1*Δ strain, but when the cellular levels of the chaperone are reduced using the tetracycline-repressible promoter, prion propagation defects become evident [88, 89]. The role of Sis1 in conjunction with the Hsp70 Ssa1 might be to bind to the prion polymers and then to recruit Hsp104 to the polymer [88, 89]. This is supported by the observation that Sis1 only affects the efficiency of

Hsp104-mediated fragmentation of Sup35 polymers in vitro if the chaperone is incorporated into the polymers along with the Hsp70 chaperone Ssa1 [90].

It is somewhat paradoxical that yeast prion polymers multiply through the action of Hsp104, which would be expected to mediate their destruction. Indeed, this action efficiently dissolves aggregates of a range of denatured proteins [91, 92] and can apparently dissolve Sup35 prion polymers to the [*prion*$^-$] form, but only when Hsp104 is overproduced in non-stressed cells [76]. Furthermore, [*PSI*$^+$] is the only native prion that can be eliminated by excess Hsp104 and this is only achieved through the artificial elevation of Hsp104 levels under conditions that do not mimic any yet described known state experienced by the yeast cell. For example, Hsp104 levels are elevated in stressed cells along with other stress-inducible chaperones including Sis1 and Ssa1 and elevating Ssa1 levels certainly reduces the effectiveness of Hsp104 to eliminate [*PSI*$^+$] in non-stressed cells [93].

The cause for the paradox and the difference between the prion and the amorphous types of aggregates is that moderate destruction of prion polymers creates new sites for polymerization, thus accelerating the polymerization through the provision of new ends. In the case of amorphous, denatured protein aggregates this does not occur presumably because amorphous aggregates cannot catalyse further polymerization.

4.2 Conformational Variants of Yeast Prions

One remarkable property of yeast prions and their mammalian counterparts is the ability to take up several different yet stable [*PRION*$^+$] forms that reflect different but stable conformational states of the prion protein. In the case of mammalian prions these are referred to as prion "strains" and can result in very distinct neuropathologies [94]. In yeast, however, to avoid confusion with the use of the term strain, which is typically used to describe different lineages of this organism, they are referred to as yeast prion "variants". Like mammalian prion strains, yeast prion variants can give rise to variant-specific differences in the [*PRION*$^+$] phenotypes.

4.2.1 Discovery of [*PSI*$^+$] Variants

[*PSI*$^+$] variants were first reported by Derkatch et al. [51, 95] who described the appearance of phenotypically distinct [*PSI*$^+$] variants when [*PSI*$^+$] cells were generated de novo by overproduction of Sup35 (Fig. 5). Essentially, two basic [*PSI*$^+$] variants exist: "strong" variants that show a high level of nonsense suppression of the *ade1-14* marker coupled with high mitotic stability and "weak" variants which show a much reduced nonsense suppression phenotype and reduced mitotic stability [51, 95, 98] (Fig. 5a). In most cases the higher levels of nonsense suppression correlate with higher prion stability and a lower relative level of soluble Sup35

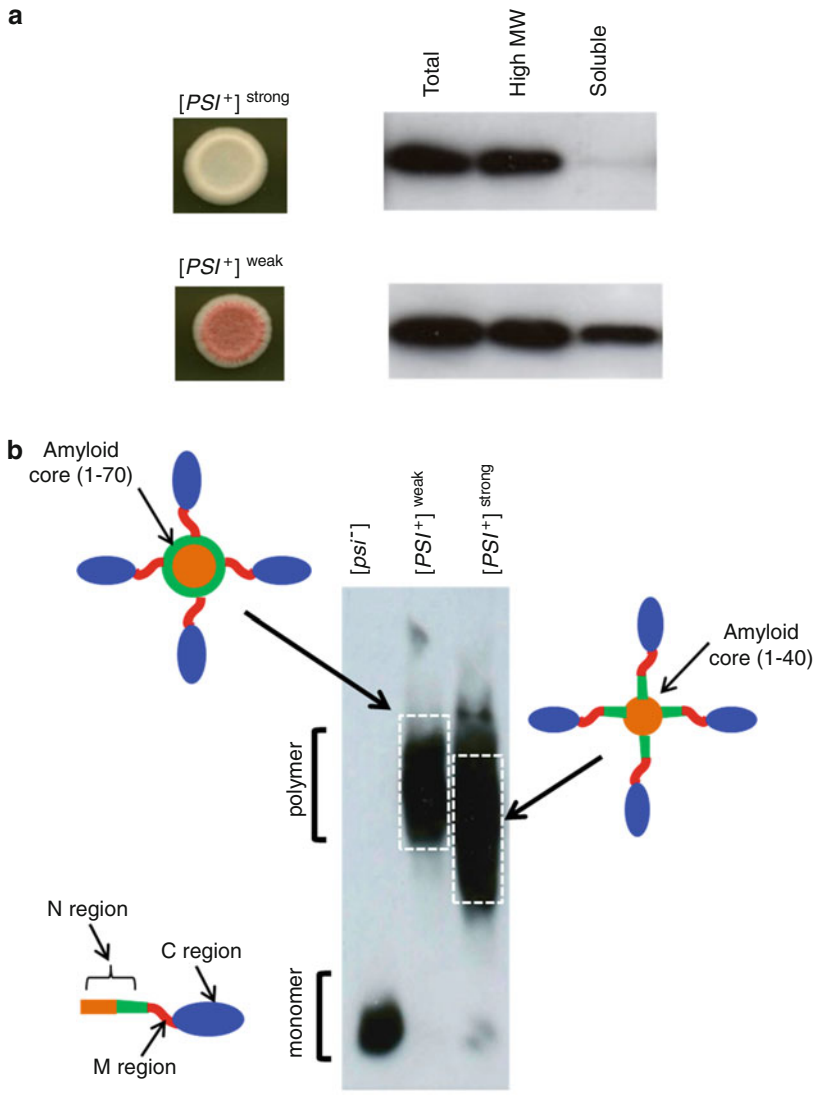

Fig. 5 The [*PSI*$^+$] prion form different conformational and phenotypically distinct weak and strong "variants". (**a**) Strong [*PSI*$^+$] variants show a high efficiency of suppression of the *ade1-14* allele (*left*) and all detectable Sup35 is present in the high molecular weight (HMW) fraction. In contrast "weak" [*PSI*$^+$] variants show a reduced efficiency of nonsense suppression consistent with a significant level of soluble and hence functional Sup35. (**b**) The strong [*PSI*$^+$] variants form lower molecular weight SDS-resistant polymers than are seen in the weak [*PSI*$^+$] variants as defined here by SDD-AGE (see Fig. 2). However the amyloid core of the Sup35 polymer in weak [*PSI*$^+$] variants span the first 70 residues of the N terminus of Sup35 whereas the core of the Sup35 amyloid formed in strong [*PSI*$^+$] variants spans the first 40 residues [96, 97]. The *-N, M* and *C* regions of Sup35 are labelled

relative to the high molecular weight polymer form of Sup35 [98]. Interestingly, when strong and weak [*PSI*$^+$] variants are combined in the same cell by genetic crosses or cytoduction, only the strong variant survives [99], most likely reflecting the propensity of "strong" [*PSI*$^+$] amyloids to take up soluble Sup35 more efficiently. The number of [*PSI*$^+$] variants is likely to be much higher than two and the terms "weak" and "strong" actually represent phenotypic groups each comprised of several subtly distinct conformational variants.

[*PSI*$^+$] variants also differ in the size of SDS-resistant Sup35 polymers; strong variants have smaller polymers of Sup35 than the weak variants [41] (Fig. 5b). This can be explained as follows, taking into account that there is no difference in the steady state levels of Sup35 in the different variants. If Sup35 polymers are smaller, their number is higher and hence Sup35 polymerisation proceeds more efficiently as the number of free ends for polymerisation is high. This results in less soluble Sup35 leading to efficient nonsense suppression due to reduced termination efficiency. The small size of prion polymers in "strong" [*PSI*$^+$] variants suggests that they are more susceptible to fragmentation by Hsp104 and indeed a mathematical model for Sup35 polymerisation [100] demonstrates that polymer size can depend solely on the rate constant for Hsp104-mediated fragmentation, i.e. the ability of amyloids to be recognised and fragmented by Hsp104 [101]. This difference in the rate constant presumably reflects differences in the structure of prion polymer.

In agreement with this model, a significant structural difference has been observed between in vitro-generated Sup35 amyloids, corresponding to weak and strong [*PSI*$^+$] variants [96, 97]. Such amyloids can be obtained by in vitro polymerization of Sup35 at different temperatures, either 4 °C, 25 °C or 37 °C. When used to transform [*psi*$^-$] cells one gets either strong or weak [*PSI*$^+$] variants emerging depending on the amyloid form and this is thought to reproduce the in vivo structure of the respective [*PSI*$^+$] variants.

How do the [*PSI*$^+$] variants differ at the structural level? This question has been studied using several different approaches. Fluorescent labelling showed that the amyloid core of the in vitro-generated Sup35 polymer comprises amino acid residues 31–86 in the strong (4 °C) fold and residues 21–121 in the weak (25 °C) fold [96]. A later study using hydrogen–deuterium exchange revealed that the amyloid core is formed by Sup35 residues 1–37 for the strong (4 °C) fold and residues 1–70 for the weak (37 °C) fold [97]. Despite the evident difference between these data, the shared conclusion is that the amyloid core is significantly smaller in the strong fold than in the weak. This conclusion is supported by the observations that the "strong" variant-inducing Sup35 amyloid fibres formed in vitro are much more fragile than the "weak" variant-inducing ones [100], while the strong fibres also show a lower thermal stability [102]. These data indicate that the "strength" of the [*PSI*$^+$] phenotype in vivo correlates with the physical weakness of the fibres. However, it is not yet clear whether good polymer fragmentation, typical of strong [*PSI*$^+$], directly relates to polymer fragility. Alternatively, the frequency of fragmentation may be determined by the ease of recognition of these polymers as a target for Hsp104-mediated fragmentation, which could be defined by the structure and/or size of the amyloid core [101].

4.2.2 Variants of Other Fungal Prions

Variants have also been described for other native yeast prions [*PIN*$^+$] [99] and [*URE3*] [103], as well as for chimeric prions comprising the Sup35 PrD from *Saccharomyces* species other than *cerevisiae,* fused to the *S. cerevisiae* C-terminal region [104, 105]. As with the [*PSI*$^+$] variants, these other yeast prion variants show differences in the relative levels of soluble prion protein and in the stability of the prion during cell division.

[*PIN*$^+$] variants were originally identified based on the efficiency with which they seeded the de novo appearance of [*PSI*$^+$] in cells engineered to overexpress Sup35 or the Sup35-PrD [99]. Four stable [*PIN*$^+$] variants were isolated that gave low, medium, high and very high frequency of [*PSI*$^+$] appearance respectively although these did not necessarily correlate with the relative levels of soluble Rnq1 [99]. Interestingly, these [*PIN*$^+$] variants showed similar efficiencies in the induced de novo induction of a second prion, [*URE3*]. A second study differentiated several [*PIN*$^+$] variants based on the relative levels of soluble Rnq1 [106]. These authors concluded that the physical basis of the differences between their [*PIN*$^+$] variants is essentially the same as described for the [*PSI*$^+$] variants with one exception. While the variant phenotype showed a strong correlation with the stability of the amyloid polymer of the Rnq1 protein – polymers from weak strains being more robust than those from a strong strain – the variant phenotypes did not necessarily correlate with the respective rates of aggregation of Rnq1 [106]. Thus, there is a clear relationship between protein conformation, the relative stability of the prion polymer with regards Hsp104-mediated fragmentation and the "strength" of a prion variant. Furthermore, these observations suggest that the number of prion variants that can arise from a single protein is likely to be high.

In the case of both the [*PIN*$^+$] variants [99, 106] and the [*URE3*] variants [103] novel in vivo reporters were developed to facilitate the differentiation between the variants at the phenotypic level. For example, for [*URE3*], a novel reporter system was developed in which the *ADE2* gene was put under control of the *DAL5* promoter [103]. In its [*prion*$^-$] form the Ure2 protein co-represses *DAL5* transcription but in [*URE3*] cells Gln3 is active, which allows the *DAL5* gene to be transcribed leading to synthesis of Ade2p (phosphoribosylaminoimidazole carboxylase). Consequently, [*URE3*] cells with such a reporter system are white or pink while [*ure3-0*] cells form red colonies. The use of the *DAL5-ADE2* system revealed [*URE3*] variants of different strength, which also corresponded to the relative levels of soluble Ure2.

4.2.3 Non-Heritable Amyloids

The spectrum of Sup35 amyloid polymers existing in vivo is not restricted to the transmissible strong and weak [*PSI*$^+$] variants described above. Some structural variants of Sup35 amyloids have also been identified that are fragmented so poorly that they fail to propagate in vivo at normal Sup35 steady state levels. These "conditionally-heritable" amyloid forms of Sup35 can be further sub-divided into

two distinct categories. Amyloids in the first category can propagate, but only when Sup35 is overproduced in the cell in which case the cells show a [*PSI*$^+$]-like nonsense suppression phenotype and propagate independently of [*PIN*$^+$] [56]. The second category are true non-heritable Sup35 amyloids with unusual properties, which exist when Sup35 is overproduced from a multicopy plasmid in the presence of the [*PIN*$^+$] prion [56]. If no selection is applied for the nonsense suppression phenotype, these amyloid forms of Sup35 are found in the majority of cells in the population, but do not give rise to the diagnostic nonsense suppression phenotype, nor are they transmitted from cell to cell.

The reason the non-heritable polymers exist is most likely due to their highly efficient seeding by Rnq1 prion polymers, since they disappear upon deletion of the *RNQ1* gene. Furthermore, a significant proportion of Rnq1 (approximately 20%) was found within SDS-resistant Sup35 polymers [56]. Non-heritable polymers are much larger than the Sup35 prion polymers seen in the weak or strong [*PSI*$^+$] variants. Consequently, it has been assumed that they are fragmented very inefficiently by Hsp104, if at all, resulting in inefficient polymerization of soluble Sup35. The result is that few polymers are present in cells expressing normal levels of Sup35. However, when Sup35 levels are artificially elevated by some 20-fold, the proportion of Sup35 found in polymers rises to ~85%. Nevertheless, the level of soluble Sup35 remaining is still sufficient for efficient translation termination and hence the nonsense suppression phenotype is not observed. The nonsense suppression phenotype can be selected for, and this is accompanied by a significant reduction in the size of the Sup35 polymers. The nonsense suppression phenotype in these cells is not heritable and is readily lost when cells are grown under non-selective conditions [56]. The Sup35 polymers observed under these selective conditions also belong to a non-heritable type, suggesting that there is also more than one structural variant of the non-heritable Sup35 amyloids.

Rnq1 can also form non-heritable amyloids when seeded by polyglutamine-derived amyloids [107]. A number of glutamine/asparagine-rich proteins have also been reported to form non-heritable amyloids in yeast, although in no case have they been shown to behave like transmissible prion amyloids [26, 107, 108]. It is possible that non-heritable amyloids appear as a matter of course when the seeded prion protein is conformationally different to the seed. Although transmissible amyloids can also appear via such cross-seeding, this would be expected to occur at a much lower frequency. For example, when Sup35 is overproduced, although non-heritable amyloids are present in almost every cell, cells containing the transmissible prion form appear at a much lower frequency, between 10^{-2} and 10^{-5} [107].

The heritable and non-heritable amyloids of yeast can therefore be considered as analogues of mammalian prions and non-infectious amyloids (e.g. polyglutamine, the amyloid beta – Aβ-protein, etc.) respectively. Prions are distinguished by the smaller size of the amyloid polymer, which improves transmissibility because they are present in larger numbers and show a higher mobility. The size of PrP prion fibrils in mammals is relatively small [109] although it has yet to be established whether the fragmentation required for PrP^{Sc} propagation arises from

chaperone-mediated cleavage or random mechanical fragmentation. Certainly no orthologue of Hsp104 has been identified in mammals. The lack of evident PrP deposits in many cases of Creutzfeldt–Jacob disease [110] may also be related to the small size of PrP polymers, since the propensity to form deposits should increase with the size of amyloids.

4.3 Importance of Protein Sequence in Prion Propagation

While many proteins have the propensity to form amyloids under specific, but not necessarily physiological, physico-chemical conditions, few proteins are able to form transmissible amyloids [111]. To establish what distinguishes these two forms of amyloid, one needs to look at the primary and secondary/tertiary structural features of prion proteins in order to identify what features are critical for the heritability property.

4.3.1 Primary Sequence

Yeast prions are able to form amyloids in vitro, and in at least three cases (Sup35, Rnq1 and Ure2) these forms, when introduced into cells lacking the respective prion, can induce the de novo formation of the corresponding heritable prion [19, 21, 53, 100]. The amyloidogenicity of a protein is highly dependent on its primary amino acid sequence and the composition of that sequence. Charge, hydrophobicity and propensity of a peptide stretch to form secondary structure will influence whether or not a protein, or at least a peptide sequence within that protein, has amyloidogenic potential [111]. The amyloids formed by fungal prions not only need to be robust enough to maintain stably the phenotype and evade the cellular protein degradation machinery, but must also be susceptible to fragmentation by the Hsp104/Hsp70/Hsp40 chaperone network in order to form propagons, the crucial heritable units that can be passed on to daughter cells.

An analysis of the known fungal prion proteins has indicated that the amino acid composition of those regions of the proteins responsible for their amyloid-based prion behaviour, i.e. the PrD, are very similar [31]. PrDs are enriched for Gln and Asn residues and, with the exception of the *P. anserina* [Het-s] prion [112], they very rarely possess charged residues. This residue bias is not unsurprising, since Gln and Asn residues are known to stabilise amyloids through the formation of polar zippers [113–115] while charged residues are likely to disrupt them through electrostatic repulsion. Asn residues in particular are important and their presence is key to the definition of a functional PrD in yeast [26]. Due to the characteristic amyloid β sheet structure, strong β-breaker residues such as Pro are largely absent from these regions [26] or are relegated to β-turns where they would not disrupt intra- or intermolecular contacts.

The salient features that have emerged from a comparison of yeast PrD sequences have been used to predict new yeast prions based solely on the analysis of protein sequence [26, 28, 29, 33]. In some other cases, e.g. New1 [52, 108], although a PrD sequence has been identified in a protein and shown to function as such when linked to the Sup35 C terminus, the full-length protein has yet to be shown to have prion-like properties. It is therefore critical – as shown by Alberti et al. [26] – that predicted prions are verified by a variety of biochemical and genetic experiments.

While the bias in the amino acid composition of yeast PrD sequences is now well established, the importance of the primary amino acid sequence for prion formation has been the subject of some debate. Several yeast PrDs (Sup35, Rnq1, New1) contain oligopeptide repeats that are important for efficient propagation of the prion form of that protein [108, 116, 117]. This is especially so for the yeast [*PSI*$^+$] prion which possesses five imperfect nonapeptide repeats with a consensus sequence PQGGYQQYN (Fig. 6). This sequence bears a striking resemblance to the five "octarepeats" found in mammalian PrP and which have the consensus sequence PHGGGWGQ.

That the oligopeptide repeats were important for [*PSI*$^+$] propagation first emerged with the discovery that a dominant negative [*PSI*$^+$] propagation mutant of Sup35 (designated *PNM2-1*) was a single amino acid substitution (Gly to Asp) in the second of the oligopeptide repeats [118]. Subsequently Liu and Lindquist [116] confirmed that the number of repeats in the Sup35-PrD is important by demonstrating that adding two additional copies of the second repeat resulted in a significant increase in the rate of de novo appearance of [*PSI*$^+$]. Such oligopeptide repeat expansion mutants of PrP have also been identified in a number of CJD patients [119, 120]. Other important features for prion stability and continued propagation were also later associated with the Sup35-PrD repeats; for example, they contribute to intermolecular interactions, either with other Sup35 molecules to strengthen the

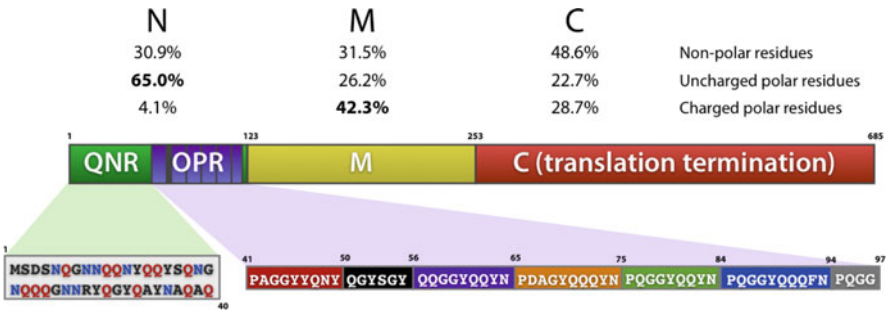

Fig. 6 The Sup35 protein has an amino terminal prion-forming domain (PrD). Sup35 can be divided in three regions: a C-terminal region with translation termination properties, a highly charged middle region of unknown function (M) and an N-terminal region which contains its PrD (1–97) and is rich in uncharged polar residues. This region can be further subdivided into the Q/N-rich region (QNR) and the oligopeptide repeat region (OPR) and these two regions are both important for propagation of the [*PSI*$^+$] prion

amyloid form [117] or with the chaperones that carry out the fragmentation of the polymers to ensure the perpetuation of the phenotype [108]. The recent conformational analysis of different variants of the [*PSI*$^+$] prion has indicated that the region spanning the oligopeptide repeats is an important contributor to the mechanical stability of the Sup35 polymers [96, 97]. This region may be important because it could provide chaperone binding sites or possess enough conformational flexibility to allow chaperone access to the amyloid core of [*PSI*$^+$] fibrils as a prerequisite for polymer fragmentation [108, 121].

The discovery that the primary sequence of both the Sup35-PrD and Ure2-PrD can be shuffled without apparently impairing the ability of the protein to form a heritable amyloid (i.e. prion) [122] would argue against the importance of such oligopeptide repeat regions and their conserved peptide motifs in prion propagation. These data are in agreement with one of the structural models put forward for yeast prions, namely the parallel in register β-sheet model proposed by Wickner and colleagues [123, 124]. This model assumes that one residue in one of the prion protein molecules will make contact with the same residue in the adjacent molecules and therefore shuffling of the sequence would have no impact on this property. This model is hard to reconcile with a second model, the β-helix model proposed by Lindquist and her colleagues [96], although the latter model also uses the "in register" principle.

The observations that deletion of some or all of the oligopeptide repeats in Sup35 leads to the loss of [*PSI*$^+$] [95, 117, 121, 125, 126] while expansion enhances de novo formation of the [*PSI*$^+$] prion [116], and that modification of a single amino acid in these repeats can block or lead to inefficient propagation of the prion [108, 118, 125] suggest an important role for this region which would be abolished when the sequence was randomised. Further investigation will be required to establish whether particular sequence motifs or structural features required for the functions described above, be they conferring conformational flexibility or chaperone binding properties, were conserved in each of the shuffled Sup35 PrDs analysed [122].

4.3.2 Secondary Structure

As with all other amyloid proteins, prion structure is dominated by highly ordered β-sheets. Spectroscopic studies on the hamster PrP prion protein using Fourier transform infrared (FTIR) and circular dichroism (CD) have shown that, while the non-prion form of the protein (PrP^C) contains 42% α-helix and very little β-sheet (3%), the prion form of the protein contains less α-helical structure (30%) and significantly more β-sheet (45%) [127]. Similar biophysical studies have been used in an attempt to obtain structural information about fungal prions, e.g. [128, 129], but the difficulties presented when trying to determine amyloid structure by conventional methods such as solution NMR or X-ray crystallography have hampered establishing the native structures of the corresponding prion proteins.

In the case of the [*PSI*$^+$] prion, it was first demonstrated that the Sup35-PrD could polymerise in vitro into amyloid-like fibrils which could then accrete more

soluble protein to their ends [17, 18, 80]. As discussed above (Sect. 4.2), the structural basis of [*PSI*$^{+}$] prion variants was unravelled by a series of demanding experiments either by chemically probing the in vitro accessibility of introduced Cys residues in the NM-region of Sup35 [96] or by combining solution NMR with hydrogen/deuterium exchange [97]. Additional studies using small peptides from the N-terminal region of Sup35 [130–133] showed very tight association of the formed β-sheets through self-complementing steric zippers, providing a rationale for the extreme stability of these amyloids [130]. These studies have shed some light on one possible mechanism for [*PSI*$^{+}$] variant formation, since the same peptides were able to form more than one complementary steric zipper [131].

What kind of secondary structure the whole Sup35 PrD forms and how it assembles into fibrils is also the subject of considerable debate. One of the theories comes from experimental evidence using solid state NMR and points towards an in-register parallel β-sheet structure which could be deduced from the intermolecular distances between ^{13}C-labelled residues in different Sup35 molecules which were adjacent in the Sup35 fibrils [123]. A different set of experiments conducted using peptide arrays and cysteine cross-linking proposed that intermolecular contacts between adjacent Sup35 molecules were exclusive to specific residues in its PrD and that contacts between molecules were made following a "head-to-head" and "tail-to-tail" fashion with a β-helix separating the "head" and "tail" domains in each molecule [96]. There is evidence supporting both the in-register parallel β-sheet model [122, 124, 134] and the β-helix model [135] and a consensus on a definitive structure is unlikely to be reached until more conclusive studies are performed.

Both the parallel in-register β-sheet structure and the β-helix structure are present in other fungal prions that have been investigated to date. In the case of the [*URE3*] [136, 137] and [*PIN*$^{+}$] prions, the PrDs of Ure2 and Rnq1 respectively take up a parallel in-register β-sheet structure [129, 138, 139], whereas studies on the *P. anserina* [Het-s] prion [129, 138] established that the Het-S prion protein can form a β-helix composed of four β-strands, two of them aligned perpendicular to the fibril axis and the remaining two stacking on top of these along the fibril axis. Whether these strands contact as head-to-head or head-to-tail has yet to be established.

4.4 Role of Cellular Factors in Prion Propagation

Studies with mammalian prion PrP have failed to identify any cellular factor, be it a protein, a co-factor or a nucleic acid species that is essential for the generation or propagation of PrP^{Sc}. This has led to the assertion that mammalian prions are "self-replicating". Fungal prions are however different; they require one or more cellular proteins in order to propagate successfully the prion form in vivo. This difference reflects in part the requirement to propagate yeast prions in a matter of

minutes in a rapidly dividing cell while mammalian prions typically multiply over many years in a non-dividing cell.

4.4.1 Hsp104 Effects on Prion Propagation

Through its role in prion polymer fragmentation, Hsp104 (Fig. 4) is the most vital of the cellular factors required for fungal prion propagation although there are a few examples where a fungal prion can apparently propagate in its absence [34, 140, 141]. The [Het-s] prion of *P. anserina* can also propagate without Hsp104, though propagon numbers are significantly reduced in cells lacking the chaperone [141]. Another amyloid able to propagate in yeast in the absence of Hsp104, is a modified form of the Sup35 protein – polyQY76 – in which a synthetic 76 residue sequence consisting of 80%Gln, 20%Tyr residues was fused to the Sup35 C terminal region. Fragmentation of polyQY76 polymers was greatly reduced in the absence of Hsp104, as evidenced by their increased size in *hsp104Δ* cells [101]. The ability to propagate a number of amyloid-forming proteins in the absence of Hsp104 suggests the presence of an additional unknown amyloid fragmenting activity, which is significantly less efficient than that of Hsp104.

While the ability of elevated levels of Hsp104 in non-stressed cells to cure [PSI^+] was the first observation linking Hsp104 to yeast prion maintenance [76], not all [PSI^+] variants are readily cured by excess of Hsp104. Strong [PSI^+] variants appear to be more resistant to overproduced Hsp104 [142] although this variant dependence has not been systematically studied to date. Curiously, [PSI^+] is almost the only prion sensitive to Hsp104 overproduction. Hsp104 does not eliminate [*URE3*] [143], [PIN^+] [51], [OCT^+] [40] and artificial forms of the [PSI^+] prion called [PSI^+]$_{PS}$ and based on the Sup35-PrD from *Pichia methanolica* fused to the C domain of Sup35 from *S. cerevisiae* [105]. However, a hybrid prion designated [RPS^+] and based on the fusion of the Rnq1-PrD to the Sup35 non-prion C domain is cured by excess Hsp104 [29] as is a similar fusion between the PrD of Mca1p and Sup35MC [33]. One possibility is that the prion loss induced by high levels of Hsp104 relies on the properties of Sup35 M domain, whose presence or alteration differentiates between [RPS^+] and [PIN^+], [PSI^+]$_{PS}$ and [PSI^+], respectively. This region appears to contribute to the efficient propagation of the natural [PSI^+] form [144]. Interestingly, Hsp104, lacking the N-terminal domain (amino acids 1–147) does not eliminate the [PSI^+] prion when overproduced, though its ability to propagate [PSI^+] and function in thermotolerance are not affected by such a deletion [145].

4.4.2 Hsp104 Structure and Function

Hsp104 is a member of the ClpB/Hsp100 family of AAA + proteins (ATPases associated with various cellular activities) that characteristically form hexameric or oligomeric ring complexes involved in the energy dependent remodelling of

macromolecules. AAA + proteins are able to perform such diverse functions as protein refolding and degradation, vesicle trafficking, DNA recombination, replication and repair, and transcriptional regulation [146]. The functional unit of Hsp104 is a hexameric ring with a central pore, through which unfolded substrate proteins are threaded using the energy of ATP hydrolysis [147, 148]. In addition to ClpB, some other bacterial Clp proteins (ClpA, ClpC, ClpX) have a similar structure, but act in proteolysis by transferring unfolded proteins to the peptidase ClpP [149].

Protein remodelling by Hsp104 and ClpB requires cooperation with the chaperones of the Hsp70 and Hsp40 families. In particular, refolding and reactivation of heat-denatured luciferase in vitro requires Hsp104, Ssa1 (Hsp70) and Ydj1 (Hsp40) [91]. Similarly, the disaggregating activity of ClpB requires DnaK (Hsp70), DnaJ (Hsp40) and GrpE [150, 151]. Both yeast Hsp104 and bacterial ClpB are only functionally compatible with their cognate Hsp70/Hsp40 chaperones.

Hsp104 and ClpB have a four-domain structure, which includes an N-terminal domain, a top ATPase domain AAA1, a long coiled-coil domain and a second ATPase domain AAA2 (Fig. 7a). A set of Hsp104/ClpB hybrids was analysed to find which part of these proteins interact with the Hsp70/Hsp40 chaperones. In yeast, only the AAA2 domain could be replaced by its bacterial counterpart without compromising [PSI^+] propagation and thermotolerance [89, 152]. A reciprocal ClpB/Hsp104 hybrid supported thermotolerance in bacteria. Intriguingly, while deletion of N-terminal domain of Hsp104 has no effect on [PSI^+] propagation, its replacement with the equivalent region from the bacterial ClpB does not allow [PSI^+] propagation [89]. These results suggest that Hsp70/40 chaperones interact with AAA1 domain and possibly with the coiled coil and N-terminal domains, and that these chaperones act upstream of Hsp104, facilitating substrate recognition.

This hypothesis received support from use of variants of ClpB and Hsp104 called BAP-ClpP and HAP-ClpP, generated by the addition of the ClpP binding site [89, 152]. These hybrid proteins place the ClpP peptidase downstream of the respective chaperones and turn them from refolding into degrading disaggregases. Degradation of aggregated protein substrates by BAP-ClpP and HAP-ClpP strictly depend on the presence of cognate Hsp70/40 system, which indicates that threading of polypeptide chains through ClpB/Hsp104 relies on an essential upstream activity of the Hsp70 system [152, 153]. The use in this system of an inactive $ClpP^{trap}$ moiety allowed the researchers to trap and identify the substrates. Using this novel approach, Tipton et al. [89] showed that the main Hsp40 chaperone acting upstream of Hsp104 in recognition of Sup35 prion aggregates is Sis1. This finding suggests that Ydj1 and other Hsp40 chaperones are not required for Hsp104-mediated fragmentation of Sup35 prion polymers. However, Ydj1 is known to interact with Hsp104, since Ydj1 is the most active Hsp40 in refolding denatured luciferase in vitro [91]. The ability of overproduced Ydj1 to interfere with [PSI^+] propagation [154] may therefore be related to its competition with Sis1 for Hsp104, Ssa1, or a prion substrate.

It is not clear whether any of the Hsp70/40 chaperones also act downstream of Hsp104 to help refold proteins extracted from prion aggregates. Such a function appears very likely, but not critical, for prion propagation.

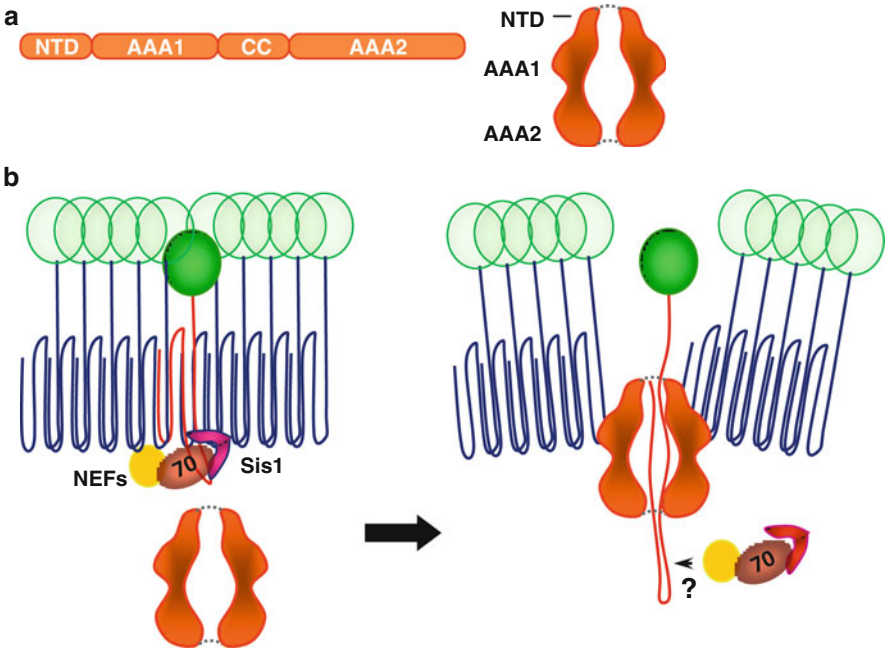

Fig. 7 The structure and function of the molecular chaperone Hsp104 and its role in prion polymer fragmentation. (**a**) The domain organisation of Hsp104. A cross-section of a hexameric functional unit of Hsp104 is shown [147] and the domains indicated are *NTD* N-terminal domain, *AAA1* and *AAA2* ATPase domains 1 and 2, *CC* coiled coil domain. (**b**) Fragmentation of prion fibres by Hsp104 and co-chaperones. Sis1 and Hsp70 (Ssa1), assisted by Hsp70 nucleotide exchange factors (NEFs), bind the prion polymer through an interaction with the PrD of a molecule in the fibre (*left*). The substrate is then transferred to Hsp104 and threaded through it until a globular functional domain is encountered at which point the substrate molecule is released. Such refolding of one molecule results in fibre fragmentation (*right*). Downstream of Hsp104, the proper folding of the "rescued" polypeptide chain may be further assisted by Hsp40s, Hsp70s and NEFs

Details of the mechanism by which Hsp104 can fragment protein polymers have begun to emerge. For example, Hsp104 can refold aggregated proteins in which the misfolded domain is located between two tightly folded domains [155] which shows that Hsp104-mediated fragmentation does not require free polypeptide ends and that two separate elements of a polypeptide chain may be threaded simultaneously through the central pore of Hsp104 hexamer (Fig. 7b). Furthermore, Hsp104 and its bacterial counterpart ClpB arrest threading of looped polypeptide segments when encountering a folded domain. This contrasts the behaviour of their proteolytic homologues (ClpC, A and X) cooperating with ClpP, which completely unfold the same polypeptides upon extraction from mixed aggregates [155]. This ability allows Hsp104 to save energy by avoiding the need to refold domains that are already correctly folded. This may also apply to yeast prion fibres where only a small part of the constituent proteins, their prion domain (PrD), require refolding by Hsp104, while the non-prion functional domains are likely to be folded correctly.

4.4.3 Hsp70/Hsp40 and Their Co-Chaperones

A growing number of proteins are known to affect both de novo prion appearance and their propagation in vivo. These proteins can interfere with prion formation or facilitate it, they can assist Hsp104 in polymer fragmentation or interfere with this process. In most cases, these activities are mediated by chaperones of the Hsp70 and Hsp40 families and the proteins that regulate functional activity and/or expression of these chaperones. The predominant role of the Hsp70/40 chaperones is not surprising given that these proteins are essential partners of Hsp104 in refolding of heat-denatured aggregated proteins [91]. In contrast, small heat shock proteins, in particular Hsp26, are also important functional partners of Hsp104 in mediating the refolding of amorphous protein aggregates [156, 157], but are not essential for prion propagation.

The Hsp70/Hsp40 chaperones are the eukaryotic equivalents of the prokaryotic DnaK/DnaJ chaperone system. In *S. cerevisiae*, cytosolic Hsp70s include four functionally redundant Ssa proteins (Ssa1–Ssa4), at least one of which is necessary for cell viability, and two non-essential Ssb proteins mainly associated with the ribosome. Hsp70 chaperones refold proteins by binding and releasing their substrates, presumably through recognition of exposed hydrophobic fragments of partially unfolded proteins. ADP-bound Hsp70 shows a high substrate affinity while in the ATP-bound state this affinity is much lower. ATP hydrolysis helps to trap a substrate on the chaperone with the ADP for ATP exchange allowing substrate release.

The intrinsic Hsp70 activities for ATP hydrolysis and ADP exchange are low and require stimulation by various co-factors. In particular, the Hsp40 chaperones bind substrates and stimulate substrate binding and ATP hydrolysis by Hsp70 [158, 159]. The yeast genome encodes ten different cytosolic Hsp40s which are thought to differ both in their specificity for substrates and the Hsp70 type for which they act as co-chaperones [160]. Three of the cytosolic Hsp40s – Ydj1, Apj1 and Sis1 – may play important roles in prion maintenance [88, 154]. The other part of the Hsp70 cycle, i.e. ADP for ATP exchange and release of substrate, is stimulated by nucleotide exchange factors (NEFs), which also participate in the mechanism of prion maintenance [161].

A possible involvement of Hsp70 chaperones in prion propagation was first highlighted by Chernoff and colleagues who found that overproduction of Ssa1 decreased [PSI^+] curing by overproduced Hsp104 [93], in contrast to overproduction of Ssb1 which further increased prion elimination [162]. Furthermore, deletion of both *SSB* genes greatly increases the frequency of the [PSI^+] formation de novo [162]. These observations have led to the proposal that Ssb proteins facilitate folding of the nascent Sup35 chains on the ribosome, thereby making them less prone to spontaneous misfolding into a heritable prion form. However, the engineered overproduction of Ssa1 and Ssb1 in non-stressed wild-type cells has no or at best a weak effect on [PSI^+] propagation [93, 162] although Ssb1 does affect the propagation of certain weak [PSI^+] variants [163]; strong effects of overexpression of either Ssa1 or Ssb1 have been reported with the artificial [PSI^+] prion derived from *Pichia* Sup35, [PSI^+]$_{PS}$[105].

One novel approach to identifying yeast prion-destabilizing factors has been to use an artificial hybrid prion $[PSI^+]_{PS}$ which consists of the putative PrD of *P. methanolica* Sup35 fused to the MC region of *S. cerevisiae* Sup35. This has provided a much more sensitive system for detecting genetic and environmental perturbations that lead to defective prion propagation and has allowed the identification of several proteins whose overproduction interferes with the $[PSI^+]_{PS}$ prion maintenance. These proteins include the Hsp70 chaperones Ssa1 and Ssb, the Hsp40s Sis1, Ydj1 and Apj1, the transcription factors Sfl1 and Ssn8, Sti1, an effector of Hsp70 ATPase activity and co-chaperone of Hsp104, and the acidic ribosomal protein Rpp0 [105, 154]. In some cases, e.g. Sfl1, Ssn8, Rpp0, the fact that their overproduction leads to an increase in the levels of molecular chaperones most likely explains their effects on $[PSI^+]_{PS}$ propagation. In addition, one of these proteins, namely Rpp0, directly interacts with Sup35 and this may also lead to the induced loss of the $[PSI^+]_{PS}$ prion (D.S. Kryndushkin, M.D. Ter-Avanesyan and V.V. Kushnirov, unpublished data). In addition, the relatively weak effects overproduction of chaperones can have on prion propagation can be explained by the very tight co-regulation of chaperone synthesis [105].

That Hsp70 has a role in $[PSI^+]$ propagation was first confirmed by the isolation of the *SSA1-21* mutant that carries a dominant mutation altering Ssa1 function, and which reduces the efficiency of $[PSI^+]$ propagation [164]. This phenotype is enhanced if the *SSA1-21* cells lack the *SSA2* (Hsp70/Ssa2) gene. The *SSA1-21* mutation causes a significant increase in the size of Sup35 aggregates though not in the size of the core polymers. The concomitant decrease in the propagon number seen explains the decreased $[PSI^+]$ stability reported [165]. The *SSA1-21* mutation is a $Leu^{483}Trp$ substitution in the substrate-binding domain of Ssa1 and leads to an almost tenfold increase in the intrinsic ATPase activity of the mutant Hsp70, an activity that is however not further stimulated by substrate or the co-chaperones Ydj1 and Sis1 [166].

Modulation of levels of the Hsp70/Hsp40s can also impact on the propagation of other prions. For example, overproduction of Ssa1 (but not of the almost identical Ssa2) leads to a loss of [*URE3*], but not $[PSI^+]$ [167] and this is also observed in cells lacking Ssa2 (but not Ssa1) [168]. [*URE3*] is also eliminated by overproduction of Ydj1 [143]. The $[PIN^+]$ prion requires the Hsp40 Sis1 for its propagation, as first suggested by the finding that deletion of a characteristic Gly-Phe-rich domain from Sis1 leads to a failure to propagate $[PIN^+]$ [169]. Subsequently it was shown that depletion of Sis1 eliminates $[PIN^+]$, as well as the $[PSI^+]$ and [*URE3*] prions [88].

NEFs that stimulate ADP–ATP exchange on Hsp70 and substrate release also play a role in yeast prion propagation; for example, the two Ssa1 co-factors Sse1 and Fes1. Overproduction of Sse1 efficiently eliminates [*URE3*], while deletion of either the *SSE1* or *FES1* gene completely blocks [*URE3*] propagation [170, 171]. Loss of Sse1, the NEF for Ssa1, also negatively impacts on $[PSI^+]$ propagation [171, 172]. Several other proteins are known to affect Hsp70 activity and include the tetratricopeptide repeat (TPR)-containing co-chaperones of Hsp90, namely Sti1 and Cns1 which stimulate Hsp70 ATPase, and a Cpr7, peptidyl-prolyl *cis–trans*

isomerase [173, 174]. Modulation of the levels of these proteins can affect the ability of elevated levels of Hsp104 to eliminate [*PSI*$^+$] in non-stressed cells; for example, depletion of Sti1 and Cpr7 inhibits the elimination of [*PSI*$^+$] by Hsp104 overproduction [175] although propagation with wild type levels of Hsp104 continues normally in cells lacking Sti1 or Cpr7 [170]. Furthermore, deletion of the *STI1* gene suppresses the Ssa1-21-mediated impairment of [*PSI*$^+$] [176].

4.4.4 Other Proteins

Proteins other than chaperones or their co-chaperones may also contribute to the process of prion propagation. One such protein is the ribosomal protein Rpp0 (see above) while overproduction of two other proteins, the endosome-associated Btn2 and its homologue Cur1, leads to elimination of [*URE3*] [177]. In the case of Btn2/Cur1, deletion of the corresponding genes leads to both an increase in the number of [*URE3*] propagons and stabilises [*URE3*] against elimination by other factors. Btn2p co-localises with Ure2 prion aggregates and, together with Cur1, Btn2p may be part of a system, similar to mammalian aggresomes, which collect aggregates and prevent their distribution to daughter cells [177].

5 De Novo Generation of Fungal Prions

The most predominant form of human CJD is the sporadic form which accounts for over 80% of the verified cases of CJD in the UK (see:www.cjd.ed.ac.uk). In such cases it is assumed that the natively folded PrP^c adopts an alternative conformational state by means of a spontaneous misfolding event. Such misfolding is stochastic in nature and can be triggered by mutation, by environmental triggers such as oxidative stress, e.g. [178] or by changes in the functioning of the cellular chaperone network, e.g. [179]. Fungal prions can also appear de novo at a detectable frequency, providing the cells already contain a second prion, namely called [*PIN*$^+$] [50, 52]. However, as with the de novo formation of mammalian PrP^{Sc}, the underlying mechanism is poorly understood in molecular terms.

Studies with the [*PSI*$^+$] prion suggest that several misfolded molecules of Sup35 may spontaneously form a catalytically active oligomer that initiates prion formation [180], although this is a rare event as the frequency with which [*PSI*$^+$] arises de novo is ~5 $\times$ 10^{-7} per generation [181]. This low frequency can be elevated by several orders of magnitude by overexpression of either full-length Sup35 or the NM region of the protein [8, 95, 182]. Consequently, as with de novo PrP^{Sc} formation, the assumption is that spontaneous formation of yeast prions arises as a consequence of a random protein misfolding event.

Studies, particularly with the [*PSI*$^+$] prion, show that the frequency of spontaneous formation of a yeast prion is regulated by environmental, cellular and epigenetic factors [183, 184]. For example, for [*PSI*$^+$] to form de novo, there is

a strong requirement for the cells to carry the [*PIN*$^+$] prion which in most laboratory strains is the prion form of the Rnq1 protein [50, 52]. The data so far available are consistent with [*PIN*$^+$] prion aggregates acting as imperfect templates on which Sup35 molecules misfold and assemble into the characteristic transmissible prion aggregates [54, 56].

Clues as to how the initial protein misfolding event translates into a stable and transmissible prion have begun to emerge. In particular, to understand better the pathway of prion induction, Tyedmers et al. [185] exploited a Sup35NM-GFP fusion protein that can be switched to a heritable prion form by wild type [*PSI*$^+$] prions. As with wild type Sup35, the Sup35NM-GFP fusion protein retains the unstructured PrD and it is this region that is believed to have a high propensity to misfold [186]. Any molecules that do misfold are targeted to a recently discovered cellular site known as the iPOD (Insoluble Protein Deposit). The iPOD is part of the quality control machinery in eukaryotic cells that is located adjacent to the vacuole and accumulates the prion forms of the Sup35, Ure2 and Rnq1 as well as oxidatively damaged proteins [185, 187]. The resulting localised concentration of misfolded proteins in a [*PIN*$^+$] cell together with the aggregated Rnq1 presumably facilitates the nucleation of prion protein polymerisation in a [*PIN*$^+$]-dependent manner. The prions are then formed via a two stage process which initially involves the formation of non-transmissible extended polymers of the prion protein followed by their fragmentation into shorter transmissible prion protein polymers [185]. Analysis of Sup35-GFP fusions in [*PSI*$^+$] cells has indicated that this form of Sup35 can form bundled fibrillar structures similar to what is seen when Sup35 is polymerised in vitro [185, 188].

5.1 Environmental Triggers

Protein folding can be influenced by a variety of environmental parameters either by directly affecting the way a protein folds and/or interacts with other proteins, or by inducing changes in the cellular levels and stoichiometry of the chaperones and other folding factors. That such changes in the environment can trigger prion formation in yeast has been demonstrated by the discovery that oxidative stress leads to a significant increase in the frequency of de novo [*PSI*$^+$] formation [184] and points to oxidative damage to proteins as being one of the key triggers of de novo prion formation [189]. Oxidative damage may also trigger de novo PrP^{Sc} formation at least in vitro [178, 190, 191]. However, not all stresses that induce [*PSI*$^+$] generation de novo cause a direct physical modification to the polypeptide. For example, Tyedmers et al. [184] showed that high salt concentrations lead to $>$50-fold increase in the appearance of [*PSI*$^+$] and in this case the presumption must be that the resulting impact on the cellular chaperone network and protein homeostasis leads to increased misfolding of a wide range of proteins, in particular those such as Sup35 that show a high propensity to misfold.

5.2 Genetic Control

Further insight into the pathway of yeast prion induction has emerged from the identification of mutants that either enhance or reduce the frequency of de novo formation of [*PSI*$^+$]. However, only one mutant of the prion protein itself has so far been identified that increases the rate of switching to the prion conformation, namely the R2E2 mutant allele of *SUP35*. This artificially constructed allele has two additional copies of one of the oligopeptide repeats in the Sup35-PrD and cells expressing this form show a significant elevation in the frequency of de novo formation of [*PSI*$^+$] [116, 184]. In contrast, naturally-occurring deletions within the Sup35 oligopeptide repeat region result in an inability to form [*PSI*$^+$] de novo [192].

Individual deletions of a number of different genes has revealed the roles played by four major classes of proteins that regulate the frequency of de novo prion formation: members of the chaperone network, components of the protein degradation machinery, especially the ubiquitin-proteasome system (UPS), cytoskeletal-associated proteins and proteins that interact with Sup35. A genome-wide screen also identified a number of other classes of protein [184] but these have yet to be investigated fully. It is perhaps not surprising that certain molecular chaperones feature amongst the proteins that regulate de novo prion formation given their role in the propagation cycle, but appears to be restricted to the Hsp70 chaperones Ssa1/2, Ssb1/2 and the Ssa co-chaperone Sse1p [183]. Inhibition of Hsp104 function by guanidine hydrochloride does not prevent the de novo formation of [*PSI*$^+$] induced by overexpression of Sup35NM or full-length Sup35 [183] which is in contrast to in vitro studies which suggest that Hsp104 promotes the polymerization of soluble full-length Sup35 into fibrils [78, 87].

The discovery that the peroxiredoxins Tsa1 and Tsa2 are important regulators of de novo prion formation in yeast has also provided direct evidence that a failure to correct oxidative damage to proteins may trigger de novo prion formation [189]. In a *tsa1 tsa2* [*psi*$^-$] [*PIN*$^+$] strain, the frequency of [*PSI*$^+$] de novo formation is significantly elevated. These Tsa1/Tsa2 proteins co-localise to ribosomes and are believed to protect Sup35 against oxidative stress-induced formation of its heritable [*PSI*$^+$] prion conformation [189]. Molecular oxygen is certainly required for [*PSI*$^+$] prion formation since growth under anaerobic conditions prevents prion formation in a *tsa1 tsa2* mutant [189]. Conversely, oxidative stress conditions induced by exposure to hydrogen peroxide elevates the rate of de novo [*PSI*$^+$] prion formation [184].

6 Why Do Fungal Prions Exist?

Years of research into protein misfolding diseases such as Alzheimer's disease, Huntington's disease or Creutzfeldt–Jakob disease have clearly established amyloids as disease associated, but not necessarily disease causing. In mammals,

amyloid accumulation as a consequence of a failure of the host's proteolytic machinery to degrade it is usually highly detrimental to the host, leading to cell death. The subsequent damage that amyloid protein can cause to tissues ultimately compromises entire cellular systems and ultimately the viability of the host. One major unresolved question in the fungal prion field is whether specific fungal prions, that form amyloids in the cell, have a positive (beneficial) or negative (disease) impact on the host.

The first indication that a yeast prion may have a beneficial effect on the host came from the finding that [PSI^+] cells are better able to withstand heat stress than otherwise isogenic [psi^-] cells [44]. Subsequently, a detailed study [45, 46] revealed that [PSI^+] cells are also better able to tolerate several different physical and chemical challenges and this led to the suggestion that cells carrying the [PSI^+] prion have a short-term survival advantage in certain ecological niches. On an evolutionary timescale, the presence of this prion may also be allowed for in the acquisition of new genetic traits. The [Het-s] prion can also be considered as conferring a benefit to *P. anserina* cells carrying it because it prevents potentially detrimental transfer of infectious cytoplasmic agents, in particular viruses, between different strains of the fungus [23, 58]. The prion properties of the underlying Het-s protein allow for a relatively easy switch between an "open" state which allows for exchange of genetic material and a "conservative" state, blocking crosses with distantly-related strains of the fungus.

As exciting as the idea of prions as evolutionarily established dynamic regulators of cell function may be, an argument can be constructed that yeast prions may in fact be deleterious to the host and can thus be considered as disease-causing [193]. At the crux of this argument is the failure to find either the [PSI^+] or [*URE3*] prion in strains of *S. cerevisiae* isolated from "natural habitats" [192, 193]. However, at least one prion does exist in naturally-occurring strains, namely [PIN^+] [192], although the phenotypic impact of this prion appears to be restricted to its promotion of the de novo formation of other prions (see above). It remains to be established whether or not the other described prions are present in strains outside the laboratory.

The potential impact of the [PSI^+] prion on host cells needs further consideration not least because nonsense suppression would be expected to be detrimental to a cell. The relatively high level of translation of stop codons, i.e. termination readthrough, could potentially lead to the generation of an aberrant proteome, full of misfolded, non-functional proteins that the cell simply would not be able to cope with. One model that can account for the apparent non-harmful effect of [PSI^+] on host cells is that there are mechanisms that operate to reduce potentially harmful readthrough of natural terminators, for example codon context [194, 195]. Alternatively it may act as an adaptive molecular switch that enables cells to explore a whole new proteome through the suppression of nonsense codons without any underlying long-term genetic change. These codons could be the native terminators with the [PSI^+]-mediated termination readthrough leading to a C-terminal extension of the encoded polypeptide chain, thereby modifying the function of the protein, possibly inhibiting its normal function. Such a phenomenon has been

noted with [*PSI*$^+$]-mediated readthrough of the *PDE2* mRNA stop codon; *PDE2* encodes a high-affinity cAMP phosphodiesterase [47]. Alternatively, the stop codon could arise as a premature termination mutation and [*PSI*$^+$]-mediated readthrough would allow the silenced gene to express a full-length, wild type protein, albeit at lower levels than normal (nonsense suppression is never 100% efficient). In either case one can envision scenarios where possessing the resulting alternate proteome could enhance the fitness of the cells containing the prion.

Amongst the most recently discovered prions are also proteins involved in transcriptional regulation, namely Mot3 ([*MOT3*$^+$]), Sfp1 ([*ISP*$^+$]) and Cyc8 ([*OCT*$^+$]) as well as the chromatin-remodelling factor Swi1 ([*SWI*$^+$]). If changes in the prion state of these proteins have an impact on gene expression, could their prionisation be activated to respond to environmental stress? In addition, the [*PIN*$^+$] prion seems to function solely to enable other prions to form de novo. Could there be a hidden stress-sensing function associated with this protein or interacting factor that could trigger its prionisation, thereby starting a cascade of events leading to the "activation" of all other prions as a stress response? Prion variants add a whole additional dimension to this hypothesis, since through them one can envisage how cells can subtly regulate the otherwise "rigid" adaptive response.

7 Conclusion

That prions exist in the fungal world is without question. Since their discovery in 1994, the evidence to support the existence of a protein-based inheritance system in yeast has become overwhelming and the number of possible prions continues to increase. The mechanism by which yeast prions can be efficiently and faithfully transmitted during mitosis and meiosis is beginning to emerge from the application of in vitro and more recently in vivo studies that fully exploit the tractability of *S. cerevisiae*. Although originally touted as a model to understand better prion biology and how animal prions form and can be transmitted, it is now evident that yeast prions may actually represent a new epigenetic system that can have a wide ranging impact on host phenotype without needing to resort to changes at the DNA level. Whether yeast prions are detrimental disease-causing entities or actually bring short-term and/or long-term benefits to the host remains a subject of much contention. Yet there remains a number of other important yet unanswered questions, the answers to which should emerge over the next decade, not least being whether *S. cerevisiae* is unique in the natural world in investing in prion-based elements to regulate its phenotype?

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