

# PRION DISEASES OF HUMANS AND ANIMALS: Their Causes and Molecular Basis

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■ **Abstract** Prion diseases are transmissible neurodegenerative conditions that include Creutzfeldt-Jakob disease (CJD) in humans and bovine spongiform encephalopathy (BSE) and scrapie in animals. Prions appear to be composed principally or entirely of abnormal isoforms of a host-encoded glycoprotein, prion protein. Prion propagation involves recruitment of host cellular prion protein, composed primarily of  $\alpha$ -helical structure, into a disease specific isoform rich in  $\beta$ -sheet structure. The existence of multiple prion strains has been difficult to explain in terms of a protein-only infections agent, but recent studies suggest that strain specific phenotypes can be encoded by different prion protein conformations and glycosylation patterns. The ability of a protein to encode phenotypic information has important biological implications. The appearance of a novel human prion disease, variant CJD, and the clear experimental evidence that it is caused by exposure to BSE has highlighted the need to understand the molecular basis of prion propagation, pathogenesis, and the barriers limiting inter-mammalian transmission. It is unclear if a large epidemic of variant CJD will occur in the years ahead.

## INTRODUCTION

### Historical Background

The prion diseases are a closely related group of neurodegenerative conditions that affect both humans and animals. They have previously been described as subacute spongiform encephalopathies, slow virus diseases, and transmissible dementias. The prototypic disease is scrapie, a naturally occurring disease affecting sheep and goats. Scrapie has been recognized in Europe for over 200 years (McGowan 1922) and is present in many countries worldwide. More recently recognized animal diseases include transmissible mink encephalopathy (Marsh 1992), chronic wasting disease of mule deer and elk (Williams & Young 1980), and bovine spongiform encephalopathy (BSE) (Wells et al 1987). The more recently described feline

spongiform encephalopathy of domestic cats (Wyatt et al 1991) and spongiform encephalopathies of a number of zoo animals (Jeffrey & Wells 1988, Kirkwood et al 1990) are also recognized as animal prion diseases.

Traditionally, human prion diseases have been classified into Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler syndrome (GSS) (also known as Gerstmann-Sträussler-Scheinker disease), and kuru. Although rare neurodegenerative disorders, affecting per annum about one person per million worldwide, these diseases have had remarkable attention focused on them recently. This is because of the unique biology of the transmissible agent or prion, and also because of fears that through dietary exposure to infected tissues, an epidemic of a newly recognized bovine prion disease, (BSE,) could pose a threat to public health.

In 1936, scrapie was demonstrated to be transmissible by inoculation between sheep (and goats) following prolonged incubation periods (Cuillé & Chelle 1936). It was assumed that some type of virus must be the causative agent, and in 1954 Sigurdsson coined the term slow virus infection. There was considerable interest in the 1950s in an epidemic, among the Fore linguistic group of the Eastern Highlands of Papua New Guinea, of a neurodegenerative disease, kuru, characterized principally by a progressive ataxia. Subsequent field work by a number of investigators suggested that kuru was transmitted during cannibalistic feasts. In 1959, Hadlow drew attention to the similarities between kuru and scrapie at the neuropathological, clinical, and epidemiological levels, leading to the suggestion that these diseases may also be transmissible (Klatzo et al 1959, Hadlow 1959). A landmark in the field was the transmission by intracerebral inoculation with brain homogenates into chimpanzees of first kuru (Gajdusek et al 1966) and then CJD (Gibbs et al 1968). Transmission of GSS followed in 1981 (Masters et al 1981). This work led to the concept of "transmissible dementias." The term Creutzfeldt-Jakob disease (CJD) was introduced in 1922 by Spielmeyer, who drew from earlier case reports of Creutzfeldt and Jakob. In subsequent years, the term was used to describe a range of neurodegenerative conditions, many of which would not meet modern diagnostic criteria for CJD. The new criterion of transmissibility allowed the diagnostic criteria for CJD to be assessed and refined. Atypical cases could be classified as CJD on the basis of their transmissibility. Both animal and human conditions share common histopathological features. The classical triad of spongiform vacuolation (affecting any part of the cerebral grey matter), neuronal loss, and astrocytic proliferation may be accompanied by amyloid plaques (Beck & Daniel 1987).

The nature of the transmissible agent in these diseases has been a subject of heated debate for many years. The understandable initial assumption that the agent must be some form of virus was challenged, however, both by the failure to directly demonstrate such a virus (or indeed any immunological response to it) and by evidence indicating that the transmissible agent showed remarkable resistance to treatment expected to inactivate nucleic acids (such as ultraviolet radiation or treatment with nucleases). As early as 1966, such findings had led to suggestions that the transmissible agent may be devoid of nucleic acid (Alper et al 1966, 1967).

They also led Griffith (1967) to suggest that the transmissible agent might be a protein. Progressive enrichment of brain homogenates for infectivity resulted in the isolation by Bolton et al (1982) of a protease-resistant sialoglycoprotein, designated the prion protein (PrP). This protein was the major constituent of infective fractions and was found to accumulate in affected brains and sometimes to form amyloid deposits. The term prion (from the first letters of proteinaceous infectious particle) was proposed (Prusiner 1982) to distinguish the infectious pathogen from viruses or viroids. Prions were defined as "small proteinaceous infectious particles that resist inactivation by procedures which modify nucleic acids" (Prusiner 1982).

The protease-resistant PrP extracted from affected brains was of 27–30 kDa and became known as PrP<sup>27–30</sup>. At the time, PrP was assumed to be encoded by a gene within the putative slow virus thought to be responsible for these diseases. However, amino acid sequencing of part of PrP<sup>27–30</sup> led to the recovery of cognate cDNA clones using an isocoding mixture of oligonucleotides. PrP<sup>27–30</sup> was demonstrated in 1985 to be encoded by a single-copy chromosomal gene rather than by a putative nucleic acid in fractions enriched for scrapie infectivity. PrP<sup>27–30</sup> is derived from a larger molecule of 33–35 kDa, designated PrP<sup>Sc</sup> (denoting the scrapie isoform of the protein) (Oesch et al 1985). The normal product of the PrP gene, however, is protease sensitive and designated PrP<sup>C</sup> (denoting the cellular isoform of the protein). No differences in amino acid sequence between PrP<sup>Sc</sup> and PrP<sup>C</sup> have been identified. PrP<sup>Sc</sup> is known to be derived from PrP<sup>C</sup> by a posttranslational process (Borchelt et al 1990, Caughey & Raymond 1991).

## Animal Prion Diseases

An increasing number of animal prion diseases are being recognized. Scrapie, a naturally occurring disease of sheep and goats, has been recognized in Europe for over 200 years and is present endemically in many countries. Accurate epidemiology is lacking, although scrapie may be common in some countries. Remarkably little is known about its natural routes of transmission. Transmissible mink encephalopathy (Marsh 1992) and chronic wasting disease of mule deer and elk (Williams & Young 1980) were described in captive animals from the 1940s onward, principally in the United States. It has more recently become apparent that chronic wasting disease may be a common condition in wild deer and elk in certain areas of Colorado (Spraker et al 1997). Again the routes of transmission are unclear (Miller et al 1998). Transmissible mink encephalopathy has occurred as infrequent epidemics among ranched mink and may result from foodborne prion exposure (Marsh et al 1991).

The appearance in UK cattle in 1986 of BSE, which rapidly evolved into a major epidemic (Wilesmith et al 1988, Anderson et al 1996), was widely attributed to transmission of sheep scrapie, endemic in the United Kingdom and many other countries, to cattle via contaminated feed prepared from rendered carcasses (Wilesmith et al 1988). However, an alternative hypothesis is that epidemic BSE resulted from recycling of rare sporadic BSE cases, as cattle were also rendered to

produce cattle feed. Whether or not BSE originated from sheep scrapie, it became clear in 1990, with the occurrence of novel spongiform encephalopathies among domestic and captive wild cats, that its host range was different from scrapie. Many new species—including greater kudu, nyala, Arabian oryx, Scimitar horned oryx, eland, gemsbok, bison, ankole, tiger, cheetah, ocelot, puma, and domestic cats—have developed spongiform encephalopathies coincident with or following the arrival of BSE. Several of these have been confirmed to be caused by a BSE-like prion strain (Bruce et al 1994, Collinge et al 1996), and it is likely that most or all of these are BSE related. More than 180,000 BSE cases have been confirmed in cattle in the United Kingdom, although the total number of infected animals has been estimated at around one million (Anderson et al 1996). BSE has since been reported in a number of other (mainly European) countries, with significant epidemics reported in Switzerland (Doherr et al 1999), Ireland, and Portugal.

## Human Prion Diseases

Human prion diseases have been traditionally classified into Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker disease (GSS), and kuru, and they can be further divided into three etiological categories: sporadic, acquired, and inherited. Acquired prion diseases include iatrogenic CJD and kuru and arise from accidental exposure to human prions through medical or surgical procedures or participation in cannibalistic feasts. Epidemiological studies do not provide any evidence for an association between sheep scrapie and the occurrence of CJD in humans (Brown et al 1987). Sporadic CJD occurs in all countries with a random case distribution and an annual incidence of one per million. Around 15% of human prion disease is inherited, and all cases to date have been associated with coding mutations in the prion protein gene (*PRNP*), of which over 20 distinct types are recognized (Collinge 1997). The inherited prion diseases can be diagnosed by *PRNP* analysis, and the use of these definitive genetic diagnostic markers has allowed the recognition of a wider phenotypic spectrum of human prion disease to include a range of atypical dementias and fatal familial insomnia (Collinge et al 1990, 1992; Medori et al 1992a,b). No such pathogenic *PRNP* mutations are present in sporadic and acquired prion disease. However, a common PrP polymorphism at residue 129, where either methionine or valine can be encoded, is a key determinant of genetic susceptibility to acquired and sporadic prion diseases, the large majority of which occur in homozygous individuals (Collinge et al 1991, Palmer et al 1991, Windl et al 1996). This protective effect of *PRNP* codon 129 heterozygosity is also seen in some of the inherited prion diseases (Baker et al 1991, Hsiao et al 1992).

The appearance in the United Kingdom in 1995 of a novel human prion disease, variant CJD (vCJD), and the experimental evidence that it is caused by the same prion strain that causes BSE in cattle (see below), has raised the possibility that a major epidemic of vCJD will occur in the United Kingdom and other countries as a result of dietary or other exposure to BSE prions (Cousens et al 1997, Ghani et al 1999, Collinge 1999). These concerns, together with those of potential iatrogenic

transmission of preclinical vCJD via medical and surgical procedures, have led to intensification of efforts to understand the molecular basis of prion propagation and to develop rational therapeutics.

Many of the key advances in understanding the pathogenesis of the prion diseases have come from study of the various forms of human prion disease. In particular, the recognition that the familial forms of the human diseases are autosomal dominant inherited conditions, associated with *PRNP* coding mutations (Owen et al 1989, Hsiao et al 1989), as well as being transmissible to laboratory animals by inoculation, strongly supported the contention that the transmissible agent, or prion, was composed principally of an abnormal isoform of prion protein.

## Clinical Features of Human Prion Disease

With advances in our understanding of their etiology, it now seems more appropriate to divide the human prion diseases into inherited, sporadic, and acquired forms, with CJD, GSS, and kuru clinicopathological syndromes placed within a wider spectrum of disease. Classical (sporadic) CJD is a rapidly progressive, multifocal dementia, usually with myoclonus. Onset usually occurs in the 45- to 75-year age group, with peak onset between 60 and 65 years. The clinical progression is typically weeks long, progressing to akinetic mutism and death often in 2–3 months. Around 70% of those afflicted die in under 6 months. Prodromal features, present in approximately one third of the cases, include fatigue, insomnia, depression, weight loss, headaches, general malaise, and ill-defined pain sensations. In addition to mental deterioration and myoclonus, frequent additional neurological features include extrapyramidal signs, cerebellar ataxia, pyramidal signs, and cortical blindness.

Kuru reached epidemic proportions among a defined population living in the Eastern Highlands of Papua New Guinea and provides by far our largest experience of acquired human prion disease (Alpers 1987). The earliest cases are thought to date back to the early part of the century. Kuru affected the people of the Fore linguistic group and their neighbors, with whom they intermarried. Kuru predominantly affected women and children (of both sexes) (Alpers 1987) and among women in affected villages was the most common cause of death. Kuru was transmitted during cannibalistic feasts when deceased relatives were consumed by their close relatives and others in the immediate community. Women and children predominantly participated in the feasts and ate the brain and internal organs, which is thought to explain the differential age and sex incidence. The epidemic is thought to have originated when a case of sporadic CJD, known to occur at random in all populations, occurred in a member of this population and was, as were most deceased individuals, eaten. The recycling of prions within this relatively isolated population led to a substantial epidemic that became the major cause of death among children and adult women. Prior to the cessation of cannibalism in the late 1950s, feasts were a common occurrence, and the multiple exposures that individual kuru patients may have had complicated precise estimates of their incubation periods.

However, studies of later cases with well-defined exposures provided more precise estimates (Klitzman et al 1984). What is the range of incubation periods seen? Very infrequent cases of kuru were recorded in children as young as 4.5 years, indicating incubation periods of this order or less. However, although it is assumed that dietary exposure to kuru was the principal route of transmission, inoculation with brain or other tissue, either via cuts or sores, or into the conjunctiva (following eye rubbing), was also likely (Alpers 1987). Because such routes of transmission in experimental animals are known to result in shorter mean incubation periods than does oral exposure, these cases of very short incubation periods for kuru may not represent oral transmission. At the other extreme, occasional cases of kuru still occur in the Fore region in patients exposed during some of the last feasts held in their villages and are consistent with incubation periods exceeding 40 years (J Whitfield, MP Alpers, & J Collinge, et al, manuscript in preparation). Mean incubation periods have been estimated to be approximately 12 years (MP Alpers, personal communication).

Kuru affects both sexes, and onset of disease has ranged from age 5 to over 60. The mean clinical duration of illness is 12 months, with a range of 3 months to 3 years; the course tends to be shorter in children. The central clinical feature is progressive cerebellar ataxia. In sharp contrast to CJD, dementia is often absent, although in the terminal stages, the faculties of many patients are obtunded (Alpers 1987).

Although prion diseases can be transmitted to experimental animals by inoculation, they are not contagious in humans. Documented case-to-case spread has occurred only by cannibalism (kuru) or following accidental inoculation with prions. Such iatrogenic routes include the use of inadequately sterilized intracerebral electrodes, dura mater, and corneal grafting, and from the use of human cadaveric pituitary-derived growth hormone or gonadotrophin. It is interesting to note that cases arising from intracerebral or optic inoculation manifest clinically as classical CJD, with a rapidly progressive dementia, whereas those resulting from peripheral inoculation frequently initially present with a progressive cerebellar ataxia, reminiscent of kuru. It is not surprising that the incubation period in intracerebral cases is short (19–46 months for dura mater grafts) compared with peripheral cases (mean estimated at around 15 years).

vCJD has a clinical presentation in which behavioral and psychiatric disturbances predominate, and in some cases there are marked sensory phenomena (notably dysaesthesiae or pain in the limbs or face) (Zeidler et al 1997, Hill et al 1999b). Initial referral is often to a psychiatrist, and the most prominent feature is depression, but anxiety, withdrawal, and behavioral changes are also frequent. Other features include delusions, emotional lability, aggression, insomnia, and auditory and visual hallucinations. In most patients, a progressive cerebellar syndrome develops, with gait and limb ataxia. Dementia usually develops later in the clinical course. Myoclonus is seen in most patients, in some cases preceded by chorea. The age at onset ranges from 16 to 51 years (mean 29 years), and the clinical course is unusually prolonged (9–35 months, median 14 months). All cases to

date are homozygous for methionine at *PRNP* codon 129 (Collinge et al 1996, Hill et al 1999b). vCJD can be diagnosed by detection of characteristic PrP immunostaining and PrP<sup>Sc</sup> on tonsil biopsy (Collinge et al 1997, Hill et al 1999b). It is important that PrP<sup>Sc</sup> is only detectable in tonsil and other lymphoreticular tissues in vCJD and not in other forms of human prion disease, indicating that it has a distinctive pathogenesis. The PrP<sup>Sc</sup> type (see below) detected on Western blot in vCJD tonsil has a characteristic pattern designated type 4t. The neuropathological appearances of vCJD are striking and consistent (Will et al 1996). Although there is widespread spongiform change, gliosis and neuronal loss, most severe in the basal ganglia and thalamus, the most remarkable feature was the abundant PrP amyloid plaques in cerebral and cerebellar cortex. These consist of kuru-like, "florid" (surrounded by spongiform vacuoles), and multicentric plaque types. The florid plaques, seen previously only in scrapie, are a particularly unusual but highly consistent feature. There is also abundant pericellular PrP deposition in the cerebral and cerebellar cortex and PrP deposition in the molecular layer of the cerebellum. Some of the features of vCJD are reminiscent of kuru (Alpers 1987), in which behavioral changes and progressive ataxia predominate. In addition, peripheral sensory disturbances are well recognized in the kuru prodrome. Kuru plaques are seen in approximately 70% of cases and are especially abundant in younger kuru patients. The observation that iatrogenic prion disease related to peripheral exposure to human prions has a more kuru-like than CJD-like clinical picture may well be relevant and would be consistent with a peripheral prion exposure in vCJD also. The relatively stereotyped clinical presentation and neuropathology of vCJD contrasts sharply with sporadic CJD. This may be because vCJD is caused by a single prion strain, and it may also suggest that a relatively homogeneous, genetically susceptible subgroup of the population with short incubation periods to BSE has been selected to date.

GSS is an autosomal dominant disorder that presents classically as a chronic cerebellar ataxia with pyramidal features, with dementia occurring later in a much more prolonged clinical course than in CJD. The mean duration is approximately 5 years, with onset usually in either the third or fourth decade. Histologically, the hallmark is the presence of multicentric PrP-amyloid plaques. Although first associated with the P102L *PRNP* mutation (Hsiao et al 1989), GSS is now recognized as a pathological syndrome associated with several different *PRNP* mutations and forms a part of the phenotypic spectrum of inherited prion disease.

The identification of one of the pathogenic *PRNP* mutations in a case with neurodegenerative disease allows diagnosis of an inherited prion disease and subclassification according to mutation (Collinge et al 1989). Over 20 pathogenic mutations have been described in two groups: (a) point mutations resulting in amino acid substitutions in PrP, or in one case production of a stop codon resulting in expression of a truncated PrP; and (b) insertions encoding additional integral copies of an octapeptide repeat present in a tandem array of five copies in the normal protein (see Figure 1). All are autosomal dominantly inherited conditions. Kindreds with inherited prion disease have been described with phenotypes of classical

CJD and GSS, and also with a range of other neurodegenerative syndromes. Some families show remarkable phenotypic variability that can encompass both CJD- and GSS-like cases as well as other cases that do not conform to either CJD or GSS phenotypes (Collinge et al 1992). Such atypical prion diseases may lack the classical histological features of a spongiform encephalopathy entirely, although PrP immunohistochemistry is usually positive (Collinge et al 1990). Progressive dementia, cerebellar ataxia, pyramidal signs, chorea, myoclonus, extrapyramidal features, pseudobulbar signs, seizures, and amyotrophic features are seen in variable combinations.

## PRION PROTEINS AND PRION PROPAGATION

### The Structural Properties of Prion Proteins

A wide body of data now supports the idea that prions consist principally or entirely of an abnormal isoform of a host-encoded protein, the prion protein (PrP), designated PrP<sup>Sc</sup> ([for a review, see Prusiner (1991)].) PrP<sup>Sc</sup> is derived from PrP<sup>C</sup> by a posttranslational mechanism (Borchelt et al 1990, Caughey & Raymond 1991). Neither amino acid sequencing nor systematic study of known covalent posttranslational modifications have shown any consistent differences between PrP<sup>C</sup> and PrP<sup>Sc</sup> (Stahl et al 1993). It is proposed that PrP<sup>Sc</sup> acts as a template that promotes the conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup> and that this conversion involves only conformational change. It is clear that a full understanding of prion propagation will require knowledge both of the structure of PrP<sup>C</sup> and PrP<sup>Sc</sup> and of the mechanism of conversion between them.

### The Conformation and Stability of PrP<sup>C</sup>

The conformation of the cellular isoform was first established by nuclear magnetic resonance (NMR) measurements made on recombinant mouse protein (Riek et al 1996). Since then, NMR measurements on recombinant hamster (James et al 1997) and human PrP (Hosszu et al 1999) show that they have essentially the same conformation; however, despite strenuous efforts, no group has yet determined the three-dimensional structure of PrP<sup>C</sup> by crystallographic methods.

Following cleavage of an N-terminal signal peptide and removal of a C-terminal peptide on addition of a glycosylphosphatidylinositol (GPI) anchor, the mature PrP<sup>C</sup> species consists of an N-terminal region of about 100 amino acids, which is unstructured in the isolated molecule in solution, and a C-terminal segment, also approximately 100 amino acids in length. The C-terminal domain is folded into a largely  $\alpha$ -helical conformation (three  $\alpha$ -helices and a short antiparallel  $\beta$ -sheet) and stabilized by a single disulphide bond linking helices 2 and 3 (Riek et al 1996). There are two asparagine-linked glycosylation sites (see Figure 2).

The N-terminal region contains a segment of five repeats of an eight-amino acid sequence (the octapeptide-repeat region), expansion of which by insertional



mutation leads to inherited prion disease. Although unstructured in the isolated molecule, this region contains a tight binding site for a single  $\text{Cu}^{2+}$  ion with a dissociation constant ( $K_d$ ) of  $10^{-14}$  M. A second tight copper site ( $K_d = 10^{-13}$  M) is present upstream of the octa-repeat region but before the structured C-domain (GS Jackson, IA Murray, LLP Hosszu, N Gibbs, JP Waltho, AR Clarke, & J Collinge, submitted for publication). These values for copper-binding affinity are some eight or nine orders of magnitude tighter than previously reported (Brown et al 1997a; Hornshaw et al 1995a,b; Stockel et al 1998). Clearly, it is possible that the unstructured N-terminal region may acquire structure following copper binding. A role for PrP in copper metabolism or transport seems likely, and disturbance of this function by the conformational transitions between isoforms of PrP could be involved in prion-related neurotoxicity.

The structured C-domain folds and unfolds reversibly in response to chaotropic denaturants, and recent work on the folding kinetics of mouse PrP<sup>C</sup> (Wildegger et al 1999) demonstrates that there are no populated intermediates in the folding reaction and that the protein displays unusually rapid rates of folding and unfolding. These findings have been reinforced by hydrogen/deuterium exchange measurements on the human protein, which show that the overall equilibrium constant describing the distribution of folded and unfolded states is the same as the protection factor (Hosszu et al 1999). This shows that no partially unfolded forms or intermediates have a population greater than the unfolded state. The data suggest that PrP<sup>Sc</sup> is unlikely to be formed from a kinetic folding intermediate, as has been hypothesized in the case of amyloid formation in other systems. In fact, on the basis of population it would be more likely that PrP<sup>Sc</sup> were formed from the unfolded state of the molecule (see Figure 3).

Inherited prion diseases may produce disease by destabilizing PrP<sup>C</sup>, which would predispose the molecule to aggregate. Alternatively, a mutation could facilitate the interaction between PrP<sup>C</sup> and PrP<sup>Sc</sup> or affect the binding of a ligand or coprotein. In order to relate the folding stability of PrP<sup>C</sup> to its propensity for forming PrP<sup>Sc</sup>, several of the human mutations have been copied into recombinant mouse protein (Liemann & Glockshuber 1999). Although this work broadly concluded that there is no absolute correlation between stability and disease, all the fully penetrant pathogenic mutations show significant destabilization, whereas nonpathogenic polymorphisms have little effect.

## Structural Studies of PrP<sup>Sc</sup>

PrP<sup>Sc</sup> is extracted from affected brains as highly aggregated, detergent-insoluble material that is not amenable to high-resolution structural techniques. However, Fourier transform infrared spectroscopic methods show that PrP<sup>Sc</sup>, in sharp contrast to PrP<sup>C</sup>, has a high  $\beta$ -sheet content (Pan et al 1993). PrP<sup>Sc</sup> is covalently indistinguishable from PrP<sup>C</sup> (Stahl et al 1993, Pan et al 1993).

During infection, the underlying molecular events that lead to the conversion of PrP<sup>C</sup> to the scrapie agent remain ill defined. The most coherent and general model

thus far proposed is that the protein, PrP, fluctuates between a dominant native state, PrP<sup>C</sup>, and a series of minor conformations, one or a set of which can self-associate in an ordered manner to produce a stable supramolecular structure, PrP<sup>Sc</sup>, composed of misfolded PrP monomers. Once a stable “seed” structure is formed, PrP can then be recruited, leading to an explosive, autocatalytic formation of PrP<sup>Sc</sup>. Such a system would be extremely sensitive to three factors: (a) overall PrP<sup>C</sup> concentration; (b) the equilibrium distribution between the native conformation and the self-associating conformation; and (c) complementarity between surfaces that come together in the aggregation step. All three of these predictions from this minimal model are manifest in the etiology of prion disease: an inversely proportional relationship between PrP<sup>C</sup> expression and prion incubation period in transgenic mice (Prusiner et al 1990, Telling et al 1995, Bueler et al 1993, Collinge et al 1995b); predisposition by relatively subtle mutations in the protein sequence (Collinge 1997); and a requirement for molecular homogeneity for efficient prion propagation (Prusiner et al 1990, Palmer et al 1991).

Little is known for certain about the molecular state of the protein that constitutes the self-propagating, infectious particle itself. There are examples of infectivity in the absence of detectable PrP<sup>Sc</sup> (Collinge et al 1995a, Wille et al 1996, Lasmezas et al 1997, Shaked et al 1999), and different strains of prions (see below) are known to differ in their degree of protease resistance. A single infectious unit corresponds to approximately  $10^5$  PrP molecules (Bolton et al 1982). It is unclear whether this indicates that a large aggregate is necessary for infectivity or, at the other extreme, whether only a single one of these PrP<sup>Sc</sup> molecules is actually infectious. This relationship of PrP<sup>Sc</sup> molecules to infectivity could simply, however, relate to the rapid clearance of prions from the brain known to occur on intracerebral challenge.

## In Vitro Production of Disease-like PrP Isoforms

Direct in vitro mixing experiments (Kocisko et al 1994, 1995; Bessen et al 1995) have been performed in an attempt to produce PrP<sup>Sc</sup>. In such experiments, an excess of PrP<sup>Sc</sup> is used as a seed to convert recombinant PrP<sup>C</sup> to a protease-resistant form (designated PrP<sup>RES</sup>). However, the relative inefficiency of these reactions has precluded determining whether new infectivity has been generated. An artificial species barrier has, however, been exploited to address this issue, and such conversion products, expected to have a different host specificity (and thus which can be bioassayed in the presence of an excess of starting material), have not shown any detectable infectivity (Hill et al 1999a). These results argue that acquisition of protease resistance by PrP<sup>C</sup> is not sufficient for the propagation of infectivity. Despite the obvious limitations of such experiments, they may represent an initial step in the generation of the infectious isoform of PrP, which requires additional, as-yet-unknown cofactors for the acquisition of infectivity.

The difficulty in performing structural studies on native PrP<sup>Sc</sup> has led to attempts to produce soluble  $\beta$ -sheet-rich forms of PrP that may be amenable to NMR or crystallographic structure determination.

It is now recognized that the adage “one sequence, one conformation” is not strictly true. Depending on solvent conditions, probably any protein chain can adopt a variety of conformations in which there is a degree of periodic order (that is, extensive regions of secondary structure). For instance, a recent systematic study of the conformations adopted by the glycolytic enzyme, phosphoglycerate kinase, shows that in different media, the chain can adopt five distinct states (Damaschun et al 1999). However, such alternative states do not have precisely and tightly packed side chains, which are the hallmark of the native state of orthodox globular proteins.

Although at physiological pH recombinant fragments of PrP unfold via a two-state mechanism, an alternative folding pathway is observed at acidic pH. Studies on recombinant human PrP residues 90–231 (Swietnicki et al 1997) and mouse PrP encompassing residues 121–231 identified a distinct, detergent-stabilized equilibrium folding intermediate at pH 4.0. Circular dichroism spectroscopy indicated this intermediate was structured with predominantly  $\beta$ -sheet topology (Hornemann & Glockshuber 1998), and it has been proposed that this may be an intermediate on the pathway to PrP<sup>Sc</sup> formation. Recent studies on a large fragment of the human prion protein (PrP<sup>91–231</sup>) have shown that at acidic pH, PrP can fold to a soluble monomer comprised almost entirely of  $\beta$ -sheet in the absence of denaturants (Jackson et al 1999). Reduction of the native disulphide bond was a prerequisite for  $\beta$ -sheet formation, and these observations of alternative folding pathways dependent on solvent pH and redox potential could have important implications for the mechanism of conversion to PrP<sup>Sc</sup>. Indeed, this monomeric  $\beta$ -sheet state was prone to aggregation into fibrils with partial resistance to proteinase K digestion, characteristic markers of PrP<sup>Sc</sup>. Although unusual for a protein with a predominantly helical fold, the majority of residues in PrP<sup>91–231</sup> have a preference for  $\beta$ -conformation (55% of non-glycine/proline residues). In view of this property, it is possible that the PrP molecule is delicately balanced between radically different folds with a high-energy barrier between them: one dictated by local structural propensity (the  $\beta$ -conformation), and one requiring the precise docking of side chains (the native  $\alpha$ -conformation). Such a balance would be influenced by mutations causing inherited human prion diseases (Collinge 1997). It is also worthy of note that individuals homozygous for valine at polymorphic residue 129 of human PrP (where either methionine or valine can be encoded) are more susceptible to iatrogenic CJD (Collinge et al 1991), and valine has a much higher  $\beta$ -propensity than does methionine.

The precise subcellular localization of PrP<sup>Sc</sup> propagation remains controversial. However, there is considerable evidence implicating either late-endosome-like organelles or lysosomes (Arnold et al 1995, Mayer et al 1992, Taraboulos et al 1992, Laszlo et al 1992). The environments of these organelles are evolved to facilitate protein unfolding at low pH prior to degradation by acid-activated proteases. It is possible that the  $\alpha$ -PrP-to- $\beta$ -PrP conversion, caused by reduction and mild acidification, is relevant to the conditions that PrP<sup>C</sup> would encounter within the cell, following its internalization during recycling (Shyng et al 1993). Such a mechanism could underlie prion propagation and account for the transmitted, sporadic, and

inherited etiologies of prion disease (Figure 3). Initiation of a pathogenic self-propagating conversion reaction, with accumulation of aggregated  $\beta$ -PrP, may be induced by exposure to a "seed" of aggregated  $\beta$ -PrP following prion inoculation, or as a rare stochastic conformational change or an inevitable consequence of expression of a pathogenic PrP<sup>C</sup> mutant that is predisposed to form  $\beta$ -PrP. It remains to be demonstrated whether such alternative conformational states of the protein are sufficient to cause prion disease in an experimental host or whether other cellular cofactors are also required.

## Normal Cellular Function of PrP

PrP is highly conserved among mammals, has been identified in marsupials (Windl et al 1995) and birds (Harris et al 1993), and may be present in all vertebrates. It is expressed during early embryogenesis and is found in most tissues in adults (Manson et al 1992). However, highest levels of expression are seen in the central nervous system, in particular in association with synaptic membranes. PrP is also widely expressed in cells of the immune system (Dodelet & Cashman 1998). As a GPI-anchored cell-surface glycoprotein, it has been speculated that it may have a role in cell adhesion or signaling processes, but its precise cellular function has remained obscure. Mice lacking PrP as a result of gene knockout (*Prnp*<sup>0/0</sup>) showed no gross phenotype (Bueler et al 1992), although they were completely resistant to prion disease following inoculation and did not replicate prions (Bueler et al 1993). However, these mice were then shown to have abnormalities in synaptic physiology (Collinge et al 1994) and in circadian rhythms and sleep (Tobler et al 1996). In particular, these mice had abnormalities of inhibitory synaptic transmission in hippocampal slices with a reduced amplitude of the maximal inhibitory postsynaptic current (IPSC) that could be isolated under standardized conditions, and a depolarizing shift in the reversal potential of the IPSC (Collinge et al 1994). This phenotype was rescued by a human PrP transgene (Whittington et al 1995). Two other groups, using different methods, notably much smaller IPSCs (minimal stimulus-evoked or spontaneous miniature IPSCs) and subphysiological temperatures, failed to replicate this result in hippocampal (Lledo et al 1996) and cerebellar (Herms et al 1995) slices. However, the latter group recently found that IPSCs can be modulated by free radicals differently in *Prnp*<sup>0/0</sup> mice (Herms et al 1999), and they proposed that this could explain the previous discrepancies due to the systematic difference in temperature between the original studies at physiological temperatures (Collinge et al 1994) and room temperature (Lledo et al 1996).

A second physiological phenotype for *Prnp*<sup>0/0</sup> mice was reported, a reduction of slow after-hyperpolarizations evoked by trains of action potentials (Colling et al 1996). This phenotype has subsequently been confirmed by another group in cerebellar slices (Herms et al 1998). It is interesting that hamsters infected with scrapie also have depressed slow after-hyperpolarizations (Barrow et al 1999). The most parsimonious mechanism for the various observations on the *Prnp*<sup>0/0</sup> mice is that responses of intracellular Ca<sup>2+</sup> to depolarization are lower than normal, presumably as a result of weakened influx and/or abnormal homeostasis.

Although none of these observations defines a molecular role for PrP<sup>C</sup>, it has been argued that PrP may act as a receptor for an as-yet-unidentified extracellular ligand. Newly synthesized PrP<sup>C</sup> is transported to the cell surface and then cycles rapidly via a clathrin-mediated mechanism, with a transit time of approximately an hour, between the surface and early endosomes (Shyng et al 1994). This type of behavior is associated with other cell-surface receptors, for instance those for transferrin and low-density lipoproteins. Potential partner proteins identified by two-hybrid screening include the laminin receptor (Rieger et al 1997). However, because of the methodology used for identification, these are of questionable relevance. PrP does not fold in the cytoplasm of cells, and thus, candidates so far identified are unlikely to interact specifically with PrP.

Although *Prnp*<sup>0/0</sup> mice are completely resistant to prion infection, reconstitution of such mice with either mouse or hamster PrP transgenes restores susceptibility in a species-specific manner (Bueler et al 1993). This then allows a reverse genetics approach in transgenic mice to study structure-function relationships in PrP by expressing truncated or mutated PrP in *Prnp*<sup>0/0</sup> mice. Expression of N-terminal deletion mutants to residue 106 was tolerated and allowed prion propagation. However, deletion beyond this led to severe ataxia and neuronal loss in the granular cell layer of the cerebellum (Shmerling et al 1998), symptoms and pathology that were completely absent in the original *Prnp*<sup>0/0</sup> mice. This has led to the hypothesis that PrP and a structural homologue compete for the same receptor or ligand. An additional gene (*Prnd*) has recently been discovered downstream of the *Prnp* locus. It encodes a 179-residue protein, with between 20% and 24% identity to PrP (depending on the species), that is a plausible candidate for the putative competitor (Moore et al 1999).

PrP in its entirety is unnecessary for prion propagation. Not only can the unstructured N-terminal 90 amino acids be deleted, so can the first  $\alpha$ -helix, the second  $\beta$ -strand, and part of helix 2. In transgenic animals, a 106-amino acid fragment of the protein comprising PrP $\Delta$ 23-88 $\Delta$ 141-176 was all that was required to confer susceptibility to and propagation of prions (Muramoto et al 1996, Supattapone et al 1999).

A number of lines of evidence argue that PrP may be a metalloprotein in vivo. It has been demonstrated that two different PrP<sup>Sc</sup> types, characteristic of clinically distinct subtypes of sporadic CJD, can be interconverted in vitro by altering the metal ion occupancy (Wadsworth et al 1999). Also copper chelators can induce spongiform change in experimental animals (Pattison & Jebbett 1971), and it has been claimed that the levels of copper in the brains of PrP-null mice are lower than in wild-type mice, although this finding has not been replicated by other workers (Brown et al 1997a, Waggoner et al 2000). Moreover, it has been reported that recombinant PrP possesses superoxide dismutase activity when refolded in the presence of high concentrations of copper chloride (5 mM) (Brown et al 1999). However, binding of copper ions was found to occur only if added to the denatured protein before refolding. PrP has also been proposed to function as a copper transport protein for internalization of copper (II) ions (Pauly & Harris 1998).

With regard to physical measurements of metal interactions, it has been shown that synthetic peptides corresponding to the octapeptide repeat region of PrP bind copper (II) ions (Hornshaw et al 1995a,b). The authors concluded that copper ions bound specifically to a peptide encompassing residues 60–91 with a  $K_d$  of 6.7  $\mu\text{M}$  and a 4:1 stoichiometry. A similar binding affinity ( $K_d$  5.9  $\mu\text{M}$ ) was reported for a longer fragment encompassing residues 23–98 (Brown et al 1997a). The stoichiometry was reported to be 5.6 coppers per peptide. Binding of metal ions to full-length recombinant hamster protein (SHaPrP<sup>29–231</sup>) has also been studied, and here the authors concluded that binding was specific for copper ions (Stockel et al 1998). Saturation of binding was reached at approximately 1.8 copper ions per PrP molecule, with an average  $K_d$  of 14  $\mu\text{M}$ . The puzzling aspect of these studies is the extremely weak binding affinity for copper (II). Binding constants in the micromolar range would lead to the conclusion that such interactions are physiologically irrelevant. Also, given the nature of the octa-peptide repeat region alone, with its five histidine side chains, one would expect copper (II) to bind with a far greater affinity. For instance, for the square planar coordination of Cu (II) by four independent imidazole groups, the effective binding affinity is  $3 \times 10^{-13}$  M, and even for simple organic oxo-acids such as malonate, the affinity is  $10^{-8}$  M (Anonymous 1986).

However, two high-affinity binding sites for divalent transition metals within the human prion protein have now been characterized, consistent with affinities seen with authentic metal binding proteins (GS Jackson, IA Murray, LLP Hosszu, N Gibbs, JP Waltho, AR Clarke, & J Collinge, submitted for publication). One is in the N-terminal octapeptide-repeat segment and has a  $K_d$  for copper (II) of  $10^{-14}$  M. Other metals ( $\text{Ni}^{2+}$ ,  $\text{Zn}^{2+}$ , and  $\text{Mn}^{2+}$ ) bind three or more orders of magnitude more weakly, with relative affinities consistent with histidine coordination. NMR and fluorescence data reveal a second site around histidines 96 and 111, a region of the molecule known to be important for prion propagation, and there are less-marked resonance shifts in the globular, structured region along helices 2 and 3. The  $K_d$  for copper (II) at this site is  $4 \times 10^{-14}$  M whereas nickel (II), zinc (II), and manganese (II) bind 6, 7, and 10 orders of magnitude more weakly, respectively, regardless of whether the protein is in its oxidized  $\alpha$ -helical ( $\alpha$ -PrP) or reduced  $\beta$ -sheet ( $\beta$ -PrP) conformation. A role for PrP in copper metabolism or transport now seems likely, and disturbance of this function by the conformational transition from  $\alpha$ - to  $\beta$ -isoforms of PrP may be involved in prion-related neurotoxicity.

## PRION STRAINS

### The Conundrum of Multiple Prion Strains

A major problem for the “protein-only” hypothesis of prion propagation has been how to explain the existence of multiple isolates, or strains, of prions. Multiple distinct strains of naturally occurring sheep scrapie were isolated in mice. Such strains are distinguished by their biological properties: They produce distinct incubation

periods and patterns of neuropathological targeting (so-called lesion profiles) in defined inbred mouse lines [for a review, see Bruce et al (1992).] As they can be serially propagated in inbred mice with the same *Prnp* genotype, they cannot be encoded by differences in PrP primary structure. Furthermore, strains can be reisolated in mice after passage in intermediate species with different PrP primary structures (Bruce et al 1994). Conventionally, distinct strains of conventional pathogen are explained by differences in their nucleic acid genome. However, in the absence of such a scrapie genome, alternative possibilities must be considered. Weissmann (1991) proposed a “unified hypothesis” where, though the protein alone was argued to be sufficient to account for infectivity, it was suggested that strain characteristics could be encoded by a small cellular nucleic acid, or “coprion.” Although this hypothesis leads to the testable prediction that strain characteristics, unlike infectivity, would be sensitive to ultraviolet irradiation, no such test has been reported. At the other extreme, the protein-only hypothesis (Griffith 1967) would have to explain how a single polypeptide chain could encode multiple disease phenotypes. Clearly, understanding how a protein-only infectious agent could encode such phenotypic information is of considerable biological interest.

## The Molecular Basis of Prion Strain Diversity

Support for the idea that strain specificity may be encoded by PrP itself was provided by study of two distinct strains of transmissible mink encephalopathy prions that can be serially propagated in hamsters, designated hyper (HY) and drowsy (DY). These strains can be distinguished by differing physiochemical properties of the accumulated PrP<sup>Sc</sup> in the brains of affected hamsters (Bessen & Marsh 1992). Following limited proteolysis, strain-specific migration patterns of PrP<sup>Sc</sup> on polyacrylamide gels were seen that related to different N-terminal ends of HY and DY PrP<sup>Sc</sup> following protease treatment and implying differing conformations of HY and DY PrP<sup>Sc</sup> (Bessen & Marsh 1994).

Recently, several human PrP<sup>Sc</sup> types have been identified that are associated with different phenotypes of CJD (Parchi et al 1996, Collinge et al 1996). The different fragment sizes seen on Western blots following treatment with proteinase K suggests that there are several different human PrP<sup>Sc</sup> conformations. However, although such biochemical modifications of PrP are clearly candidates for the molecular substrate of prion strain diversity, it is necessary to be able to demonstrate that these properties fulfill the biological properties of strains, in particular that they are transmissible to the PrP in a host of both the same and different species. This has been demonstrated in studies with CJD isolates, with both PrP<sup>Sc</sup> fragment sizes and the ratios of the three PrP glycoforms (diglycosylated, monoglycosylated, and unglycosylated PrP) maintained on passage in transgenic mice expressing human PrP (Collinge et al 1996). Furthermore, transmission of human prions and bovine prions to wild-type mice results in murine PrP<sup>Sc</sup> with fragment sizes and glycoform ratios that correspond to the original inoculum (Collinge et al 1996). Variant CJD is associated with PrP<sup>Sc</sup> glycoform ratios that are distinct from those seen in classical CJD. Similar ratios are seen in BSE in cattle

and BSE when transmitted to several other species (Collinge et al 1996). These data strongly support the protein-only hypothesis of infectivity and suggest that strain variation is encoded by a combination of PrP conformation and glycosylation. Furthermore, polymorphism in PrP sequence can influence the generation of particular PrP<sup>Sc</sup> conformers (Collinge et al 1996). Transmission of PrP<sup>Sc</sup> fragment sizes from two different subtypes of inherited prion disease to transgenic mice expressing a chimeric human mouse PrP has also been reported (Telling et al 1996). As PrP glycosylation occurs before conversion to PrP<sup>Sc</sup>, the different glycoform ratios may represent selection of particular PrP<sup>C</sup> glycoforms by PrP<sup>Sc</sup> of different conformations. According to such a hypothesis, PrP conformation would be the primary determinant of strain type, with glycosylation being involved as a secondary process. However, because it is known that different cell types may glycosylate proteins differently, PrP<sup>Sc</sup> glycosylation patterns may provide a substrate for the neuropathological targeting that distinguishes different prion strains (Collinge et al 1996). Particular PrP<sup>Sc</sup> glycoforms may replicate most favorably in neuronal populations, with a similar PrP glycoform expressed on the cell surface. Such targeting could also explain the different incubation periods that also discriminate strains, since targeting of more critical brain regions, or regions with higher levels of PrP expression, might be expected to produce shorter incubation periods. Further supportive evidence for the involvement of PrP glycosylation in prion strain propagation has come from the study of transgenic mice expressing PrP with mutations interfering with N-linked glycosylation (DeArmond et al 1997).

Recent work has shown strain-specific protein conformation to be influenced by metal binding to PrP<sup>Sc</sup> (Wadsworth et al 1999). Two different human PrP<sup>Sc</sup> types, seen in clinically distinct subtypes of classical CJD, can be interconverted *in vitro* by altering the metal-ion occupancy. The dependence of PrP<sup>Sc</sup> conformation on the binding of copper and zinc represents a novel mechanism for posttranslational modification of PrP, and for the generation of multiple prion strains. This finding may also explain differences in molecular classification of classical CJD. Collinge et al (Collinge et al 1996) described three PrP<sup>Sc</sup> types among cases of sporadic and iatrogenic CJD and a distinctive type 4 pattern in all cases of variant CJD. An earlier study (Parchi et al 1996) of PrP<sup>Sc</sup> types in classical CJD had described only two types of PrP<sup>Sc</sup>, and these authors have argued that the types 1 and 2 of Collinge et al (1996) correspond to their type 1, whereas the type 3 pattern of Collinge et al corresponds to their type 2 (Parchi et al 1997). However, these authors concede a degree of heterogeneity in their type 1 cases (Parchi et al 1996). In a large-scale study of PrP<sup>Sc</sup> types in CJD in conjunction with the UK National CJD Surveillance Unit, we demonstrated that patients classified as type 1 and type 2 using our criteria have distinct disease phenotypes, confirming the validity of our molecular classification. Type 1 human CJD is a distinct human prion disease with an aggressive clinical course and remarkably short clinical duration (Wadsworth et al 1999). In the presence of the metal chelators, human PrP<sup>Sc</sup> types 1 and 2 produce a similar-sized fragment after proteinase K digestion, designated type 2-.



It is possible, therefore, that the discrepancy between these two different molecular classification systems may be explained by differing methodologies.

Molecular strain typing of prion isolates can now be applied to molecular diagnosis of vCJD (Collinge et al 1996, Hill et al 1997) and to produce a new classification of human prion diseases, with implications for epidemiological studies investigating the etiology of sporadic CJD. Such methods allow strain typing to be performed in days rather than the 1–2 years required for classical biological strain typing. This technique may also be applicable to determining whether BSE has transmitted to other species (Collinge et al 1996), for instance to sheep (Hill et al 1998, Kuczius et al 1998, Hope et al 1999), and thereby poses a threat to human health.

Such ability of a single polypeptide chain to encode information specifying distinct phenotypes of disease raises intriguing evolutionary questions. Do other proteins behave in this way? The novel pathogenic mechanisms involved in prion propagation may be of far wider significance and may be relevant to other neurological and nonneurological illnesses; indeed, other prion-like mechanisms have now been described (Milner & Medcalf 1991), and the field of yeast and fungal prions has emerged (Wickner & Masison 1996, Wickner 1997).

## PRION TRANSMISSION BARRIERS

### The “Species Barrier”

Transmission of prion diseases between different mammalian species is restricted by a “species barrier” (Pattison 1965). On primary passage of prions from species A to species B, usually not all inoculated animals of species B develop disease. Those that do have much longer and more variable incubation periods than those that are seen with transmission of prions within the same species, where typically all inoculated animals would succumb within a relatively short, and remarkably consistent, incubation period. On second passage of infectivity to further animals of species B, transmission parameters resemble within-species transmissions, with most, if not all, animals developing the disease with short and consistent incubation periods. Species barriers can therefore be quantitated by measuring the fall in mean incubation period on primary and second passage or, perhaps more rigorously, by a comparative titration study. The latter involves inoculating serial dilutions of an inoculum in both the donor and host species and comparing the mean lethal doses ( $LD_{50}$ ) obtained. The effect of a very substantial species barrier (for instance that between hamsters and mice) is that few, if any, animals succumb to disease on primary passage, and only then at incubation periods approaching the natural lifespan of the species concerned.

Early studies of the molecular basis of the species barrier argued that it resided principally in differences in PrP primary structure between the species from which the inoculum was derived and the inoculated host. Transgenic mice expressing

hamster PrP were, unlike wild-type mice, highly susceptible to infection with Sc237 hamster prions (Prusiner et al 1990). That most sporadic and acquired CJD occurred in individuals homozygous at *PRNP* polymorphic codon 129 supported the view that prion propagation proceeded most efficiently when the interacting PrP<sup>Sc</sup> and PrP<sup>C</sup> were of identical primary structure (Collinge et al 1991, Palmer et al 1991). However, it has been long recognized that prion strain type affects ease of transmission to another species. It is interesting that with BSE prions, the strain component to the barrier seems to predominate, with BSE not only transmitting efficiently to a range of species but also maintaining its transmission characteristics even when passaged through an intermediate species with a distinct PrP gene (Bruce et al 1994). For instance, transmission of CJD prions to conventional mice is difficult, with few if any inoculated mice succumbing after prolonged incubation periods, consistent with a substantial species barrier (Collinge et al 1995b, Hill et al 1997). In sharp contrast, transgenic mice expressing only human PrP are highly susceptible to CJD prions, with 100% attack rate and consistent short incubation periods that are unaltered by second passage, consistent with a complete lack of species barrier (Collinge et al 1995b). However, vCJD prions (again comprising human PrP of identical primary structure) transmit much more readily to wild-type mice than do classical CJD prions, whereas transmission to transgenic mice is relatively less efficient than with classical CJD (Hill et al 1997). The term species barrier does not seem appropriate to describe such effects and “species-strain barrier” or simply “transmission barrier” may be preferable (Collinge 1999). Both PrP amino acid sequence and strain type affect the three-dimensional structure of glycosylated PrP, which will presumably, in turn, affect the efficiency of the protein-protein interactions thought to determine prion propagation.

### Prion Transmission Barrier: Molecular Basis

Mammalian PrP genes are highly conserved. Presumably only a restricted number of different PrP<sup>Sc</sup> conformations (that are highly stable and can therefore be serially propagated) will be permissible thermodynamically and will constitute the range of prion strains seen. PrP glycosylation may be important in stabilizing particular PrP<sup>Sc</sup> conformations. Although a significant number of different such PrP<sup>Sc</sup> conformations may be possible among the range of mammalian PrPs, only a subset of these would be allowable for a given single mammalian PrP. Substantial overlap between the favored conformations for PrP<sup>Sc</sup> derived from species A and species B might therefore result in relatively easy transmission of prion diseases between these two species, while two species with no preferred PrP<sup>Sc</sup> conformations in common would have a large barrier to transmission (and indeed transmission would necessitate a change of strain type). According to such a model of a prion transmission barrier, BSE may represent a thermodynamically highly favored PrP<sup>Sc</sup> conformation that is permissive for PrP expressed in a wide range of different species, accounting for the remarkable promiscuity of this strain in mammals. Contribution of other components to the species barrier are possible and

may involve interacting cofactors that mediate the efficiency of prion propagation, although no such factors have yet been identified.

Recent data have further challenged our understanding of transmission barriers (Hill et al 2000). The assessment of species barriers has relied on the development of a clinical disease in inoculated animals. On this basis there is a highly efficient barrier limiting transmission of hamster Sc237 prions to mice. Indeed, the hamster scrapie strain Sc237 (Scott et al 1989) [which is similar to the strain classified as 263K (Kimberlin & Walker 1977, 1978)] is regarded as nonpathogenic for mice [with no clinical disease in mice observed for up to 735 days postinoculation (Kimberlin & Walker 1978)] and was used in studies of species barriers in transgenic mice (Kimberlin & Walker 1979, Scott et al 1989, Prusiner et al 1990). It was demonstrated that transgenic mice expressing hamster PrP (in addition to endogenous mouse PrP), in sharp contrast to conventional mice, were highly susceptible to Sc237 hamster prions, with consistent, short incubation periods that were inversely correlated to hamster PrP expression levels (Scott et al 1989, Prusiner et al 1990). It is important, however, that these studies defined transmission using clinical criteria and did not report PrP<sup>Sc</sup> levels and types, or prion titers, in the brains of clinically unaffected animals. However, although not developing a clinical disease, and indeed living as long as mock-inoculated mice, Sc237-inoculated mice may accumulate high levels of prions in their brains (Hill et al 2000). Previous studies on the species barrier between hamsters and mice (using the Sc237 or 263K strain) did not report whether PrP<sup>Sc</sup> and/or infectivity were present in clinically unaffected animals (Scott et al 1989, Prusiner et al 1990) or have attempted passage from mice only up to 280 days postinoculation (Kimberlin & Walker 1978). The barrier to primary passage appears in this case to be to the development of rapid neurodegeneration and the resulting clinical syndrome rather than a barrier to prion propagation itself.

## BOVINE SPONGIFORM ENCEPALOPATHY AND RISKS TO PUBLIC HEALTH

### Variant CJD

A novel form of human prion disease, variant CJD (vCJD), was recognized in the United Kingdom in 1996 (Will et al 1996) and implied the arrival of a new risk factor for CJD (Collinge & Rossor 1996). These epidemiological studies argued for a link with BSE, and this was strongly supported by molecular strain typing studies (Collinge et al 1996). All cases of vCJD are associated with type 4 PrP<sup>Sc</sup>. Type 4 PrP<sup>Sc</sup> has a high proportion of the diglycosylated form of PrP<sup>Sc</sup> and is distinct from the PrP<sup>Sc</sup> types seen in classical CJD (types 1–3), with differing fragment sizes following proteinase K digestion. Also, types 1–3 are associated with a high proportion of monoglycosylated PrP<sup>Sc</sup>. The glycoform ratios of proteinase K-digested PrP<sup>Sc</sup> in vCJD were closely similar, however, to those seen in BSE

passed in a number of mammalian species. Furthermore, when prions isolated from either bovine brain or human brain are transmitted to experimental mice, PrP<sup>Sc</sup> isolated from the infected hosts is indistinguishable, either by site of proteinase K cleavage or by glycoform ratio (Collinge et al 1996). In addition, vCJD and BSE show closely similar transmission properties in both transgenic and conventional mice, with indistinguishable neuropathology in both transgenic mice and a variety of inbred strains of mice (Hill et al 1997, Bruce et al 1997). That vCJD is human BSE is therefore supported by compelling experimental data. Moreover, it was recently found that vCJD can be further distinguished by the detection of PrP<sup>Sc</sup> in the lymphoreticular system (Hill et al 1999b), a tissue distribution specific to vCJD.

## Modeling the Transmission Barrier Between Cattle and Humans

The species barrier between cattle BSE and humans cannot be directly measured, but it can be modeled in transgenic mice expressing human PrP<sup>C</sup>, which produce human PrP<sup>Sc</sup> when challenged with human prions (Collinge et al 1995b). When such mice, expressing both human PrP valine 129 (at high levels) and mouse PrP, are challenged with BSE, three possibilities could be envisaged: These mice could produce human prions, murine prions, or both. In fact, only mouse prion replication could be detected. Although there are caveats with respect to this model, particularly that human prion propagation in mouse cells may be less efficient than that of mouse prions, this result would be consistent with the bovine-to-human barrier being higher than the bovine-to-mouse barrier for this *PRNP* genotype. In the second phase of these experiments, mice expressing only human PrP were challenged with BSE. Although CJD isolates transmit efficiently to such mice at approximately 200 days, only infrequent transmissions at over 500 days were seen with BSE, consistent with a substantial species barrier for this human *PRNP* genotype (Hill et al 1997). The *PRNP* valine 129 genotype was studied initially in attempts to produce an animal model of human prion disease, as this genotype was over-represented among early cases of iatrogenic CJD (Collinge et al 1991), which suggests increased susceptibility or shorter incubation periods in this genotype. However, it is important to repeat these studies in mice expressing only human PrP methionine 129 and in heterozygotes. So far, BSE appears to have transmitted only to humans of *PRNP* codon 129 methionine homozygous genotype (Collinge et al 1996; Hill et al 1999b).

## Predictions of Epidemic Size

BSE can be readily transmitted to mice with most, if not all, inoculated animals succumbing to disease on primary passage (a high “attack rate”). This relatively modest species barrier has been formally measured by comparative titration studies of the same BSE isolate by intracerebral inoculation into cattle and mice. It indicates an approximately 1000-fold barrier (i.e. it takes 1000 times more BSE to

kill a mouse than a cow) (Wells et al 1998). The effect of this barrier on incubation periods is to increase mean incubation periods by approximately threefold and to dramatically increase the range of incubation periods seen.

Such experiments are usually performed using the most efficient, intracerebral, route of transmission. A formal titration of BSE in mice to determine an oral LD<sub>50</sub> has not been reported. However, oral challenge with approximately 10 g of BSE-affected cow brain killed the majority of exposed mice (Barlow & Middleton 1990). If the bovine-to-human species barrier were similar to that for mice, it would suggest an oral LD<sub>50</sub> in humans of an order of magnitude also similar to that for mice (approximately 10 g). Clearly, it is hoped that the species barrier limiting transmission of BSE to humans will be of a far higher order. However, if we assume a pessimistic scenario, that the barrier is similar (and it remains possible it could be lower), extrapolation with the known incubation periods in the acquired human prion diseases, such as growth hormone-related iatrogenic CJD or kuru, where transmission does not involve a species barrier (and mean incubation periods are approximately 10–15 years), would suggest mean incubation periods of BSE in humans of perhaps 30 years or more, and a range extending from 10 years to, or exceeding, a normal human lifespan. Such estimations (Collinge 1999), based on extensive experience of transmission studies across species from many research groups over several decades, suggest—only 4 years after recognition of vCJD—the need for caution with respect to optimistic assessments of likely human BSE epidemic size.

### Iatrogenic Transmission of vCJD Prions

Considerable concern has been expressed that blood and blood products from asymptomatic donors incubating vCJD may pose a risk for the iatrogenic transmission of vCJD. Reports of infectivity of blood from patients with classical CJD are infrequent and have been questioned (Brown 1995). Infectivity of blood from patients in the clinical phase of vCJD is unknown. However, PrP<sup>Sc</sup> is consistently found in the lymphoreticular system in vCJD (Hill et al 1999b), lymphocytes express significant levels of PrP<sup>C</sup> (Cashman et al 1990), and, in mice, B lymphocytes (although not necessarily expressing PrP<sup>C</sup>) are required for prion neuroinvasion following peripheral inoculation (Klein et al 1997, 1998; Collinge & Hawke 1998). UK policy is now to leucodeplete all whole blood, a practice already in use (for other health reasons) in some countries, and to acquire plasma for plasma products from outside the United Kingdom.

A further possible route of transmission of vCJD is via contaminated surgical instruments. Iatrogenic transmission of classical CJD via neurosurgical instruments has been reported (Bernoulli et al 1977), and normal hospital sterilization procedures are not likely to completely inactivate prions. Recent evidence suggests that classical CJD may also be transmitted by other surgical procedures (Collins et al 1999). Although in the United Kingdom all surgical instruments used on patients with suspected CJD are quarantined and not reused unless an alternate nonprion

diagnosis is unequivocally confirmed, the extensive lymphoreticular involvement in vCJD, which is likely to be present from a relatively early preclinical stage, raises the possibility that instruments could be contaminated in particular during those procedures that involve contact with lymphoreticular tissues. This includes the common procedures of tonsillectomy, appendicectomy, and lymph node and gastrointestinal biopsy. Recent studies have demonstrated that prions can adhere easily to metal surfaces, and prion-contaminated metal wires are an efficient vehicle for experimental transmission of prion disease (Zobeley et al 1999).

## PRION NEURODEGENERATION AND POTENTIAL THERAPEUTIC APPROACHES

### Cell Death and Prion Disease

The precise molecular nature of the infectious agent and the cause of neuronal cell death remain unclear. The current working hypothesis is that an abnormal isoform of PrP is the infectious agent, and to date, the most highly enriched preparations contain 1 infectious unit per  $10^5$  PrP monomers (Bolton et al 1982). Various hypotheses have been proposed to explain the mechanism of spongiform change and neuronal cell loss. These have included direct neurotoxic effects from a region of the prion protein encompassing residues 106–126 (Forloni et al 1993, Tagliavini et al 1993, Brown et al 1994) to increased oxidative stress in neurones as a result of PrP<sup>C</sup> depletion, which has been proposed to function as an antioxidant molecule (Brown et al 1997b). Neurotoxicity of PrP 106–126 is, however, controversial (Kunz et al 1999). It has also been suggested that PrP<sup>C</sup> plays a role in regulating apoptosis, with disturbance of normal cellular levels of PrP during infection leading to cell death (Kurschner & Morgan 1995, 1996). Certainly there have been numerous recent reports of apoptotic cells being identified in the neuronal tissue of prion disease brains (Williams et al 1997). Although PrP<sup>C</sup> expression is required for susceptibility to the disease, a number of observations argue that PrP<sup>Sc</sup>, and indeed prions (whether or not they are identical), may not themselves be highly neurotoxic. Prion diseases in which PrP<sup>Sc</sup> is barely or not detectable have been described (Medori et al 1992a, Collinge et al 1995a, Hsiao et al 1990, Lasmezas et al 1997). Mice with reduced levels of PrP<sup>C</sup> expression have extremely high levels of PrP<sup>Sc</sup> and prions in the brain and yet remain well for several months after their wild-type counterparts succumb (Bueler et al 1994). Conversely, Tg20 mice, with high levels of PrP<sup>C</sup>, have short incubation periods and yet produce low levels of PrP<sup>Sc</sup> after inoculation with mouse prions (Fischer et al 1996). In addition, brain grafts producing high levels of PrP<sup>Sc</sup> do not damage adjacent tissue in PrP knockout (*Prnp*<sup>0/0</sup>) mice (Brandner et al 1996). The cause of neurodegeneration in prion diseases remains unclear. It remains possible that prion neurodegeneration is related, at least in part, to loss of function of PrP<sup>C</sup>. That *Prnp*<sup>0/0</sup> mice [other than those associated with overexpression of the *Prnp*-like gene *Dpl* (Moore et al 1999)] do not develop

neurodegeneration could be due to compensatory adaptations during neurodevelopment. Complete or near-complete ablation of PrP expression in adult mice using conditional gene expression methods has not yet been achieved. A recent study has demonstrated that mice inoculated with Sc237 hamster prions replicate prions to high levels in their brains but do not develop clinical signs of prion disease during their normal lifespan, arguing that PrP<sup>Sc</sup> and indeed prions (whether or not they are identical) may not themselves be highly neurotoxic (Hill et al 2000). An alternative hypothesis for prion-related neurodegeneration is that a toxic, possibly infectious, intermediate is produced in the process of conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup>, with PrP<sup>Sc</sup>, present as highly aggregated material, being a relatively inert end product. The steady state level of such a toxic monomeric or oligomeric PrP intermediate could then determine rate of neurodegeneration. One possibility is that Sc237-inoculated mice propagate prions very slowly and that such a toxic intermediate is generated at extremely low levels that are tolerated by mice (Hill et al 2000).

## Approaches to Therapeutics

The prion diseases are now among the best understood of the degenerative brain diseases, and the development of rational treatments is appearing realistic. Various compounds, some known to bind PrP<sup>Sc</sup>, including Congo red (Ingrosso et al 1995), polyene antibiotics (Pocchiari et al 1987), anthracycline (Tagliavini et al 1997), dextran sulphate, pentosan polysulphate and other polyanions (Ehlers & Diringer 1984, Farquhar & Dickinson 1986, Kimberlin & Walker 1986), and  $\beta$ -sheet breaker peptides (Soto et al 2000), have been shown to have limited effects in animal models of prion disease. Unfortunately, most show a significant effect only if administered long before clinical onset (in some cases with the inoculum) and/or are impractical treatments because of toxicity or bioavailability.

The precise molecular events that bring about the conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup> and the molecular nature of the neurotoxic species remain ill defined, a fact that might seem to preclude screening for compounds that inhibit the process. However, any ligand that selectively stabilizes the PrP<sup>C</sup> state will prevent its rearrangement and might reasonably be expected to block prion replication (and presumably production of any putative toxic intermediate forms of PrP on the pathway to PrP<sup>Sc</sup> formation). Such an approach has recently been applied to block p53 conformational rearrangements, which are involved in tumorigenesis (Foster et al 1999). Advances in therapeutics will have to be matched by advances in early diagnosis of prion disease to provide effective intervention before extensive neuronal loss has occurred.

## CONCLUDING REMARKS

Prion diseases appear to be diseases of protein conformation, and elucidating their precise molecular mechanisms may, in addition to allowing us to progress with

tackling key public health issues posed by vCJD, be of far wider significance in pathobiology. It is of considerable interest that many of the more common neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases, and the polyglutamine repeat disorders (such as Huntington's disease) are also associated with abnormal protein aggregates. In addition, the apparent ability of a single polypeptide chain to encode information and specify distinctive phenotypes is unprecedented. It seems likely that evolution will have used this mechanism in many other ways.

Although the protein-only hypothesis of prion propagation is supported by compelling experimental data and now appears also able to encompass the phenomenon of prion strain diversity, the goal of the production of prions in vitro remains. PrP<sup>Sc</sup>-like forms of PrP have recently been produced from purified recombinant material, but as yet none have been shown in experimental animals to be capable of producing disease that can be serially propagated. Success in such an endeavor would not only prove the protein-only hypothesis, it would also serve as the essential model by which the mechanism of prion propagation can be understood in molecular detail.

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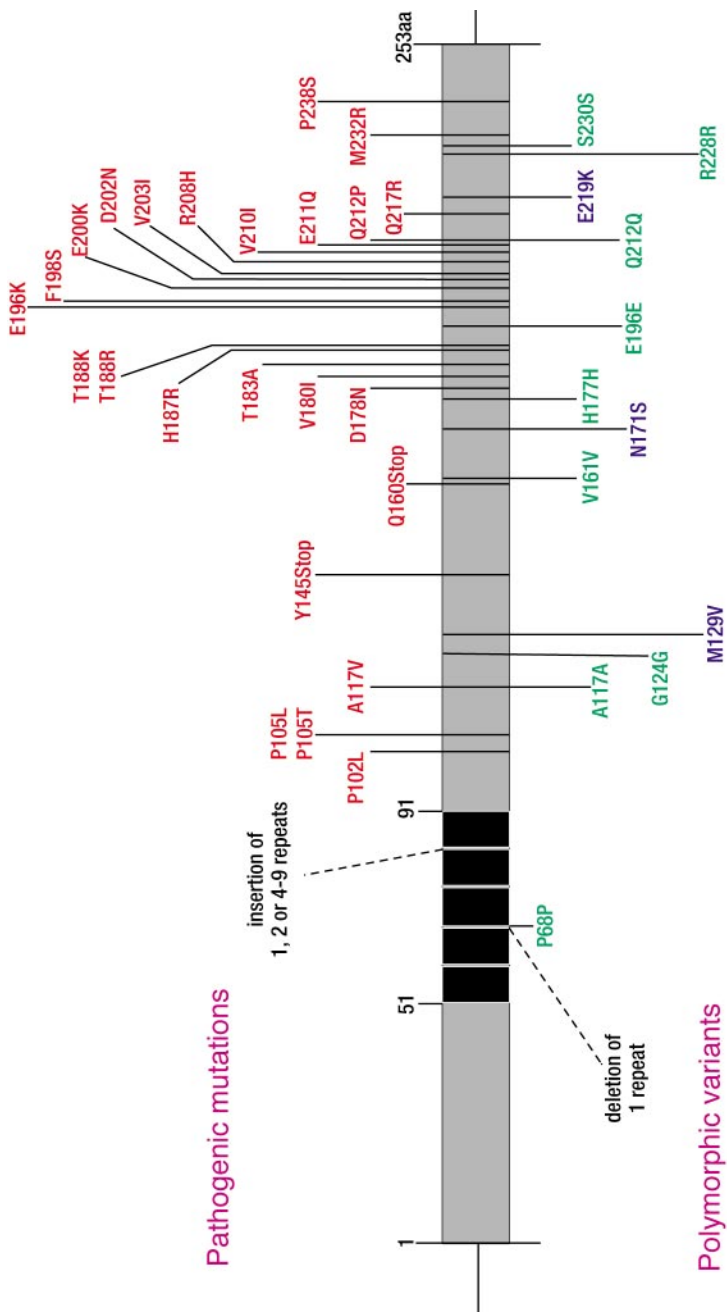
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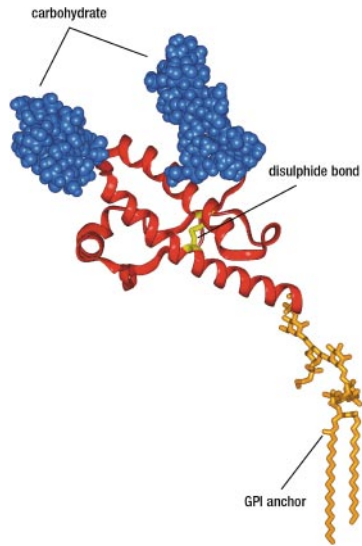
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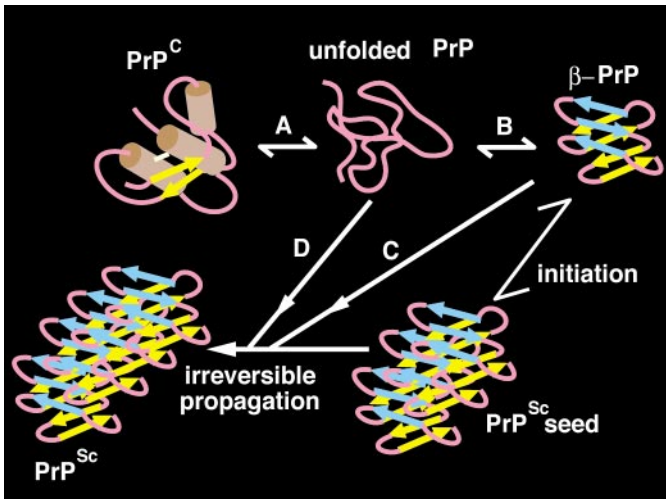




**Figure 1** Pathogenic mutations and polymorphic variants of the human prion protein.



**Figure 2** Model of glycosylated human prion protein indicating positions of N-linked glycans (in blue), the single disulphide bond joining helices 2 and 3, and the glycosylphosphatidylinositol anchor to the outer surface of cell membrane.



**Figure 3** Possible mechanism for prion propagation. Largely  $\alpha$ -helical cellular isoforms of the prion protein ( $\text{PrP}^{\text{C}}$ ) proceed via an unfolded state (A) to refold into a largely  $\beta$ -sheet form,  $\beta$ -PrP (B).  $\beta$ -PrP is prone to aggregation in physiological salt concentrations. Prion replication may require a critical “seed” size. Further recruitment of  $\beta$ -PrP monomers (C) or unfolded PrP (D) then occurs as an essentially irreversible process.