Molecular Mechanisms of Prion Pathogenesis

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Abstract

Prion diseases are infectious neurodegenerative diseases occurring in humans and animals with an invariably lethal outcome. One fundamental mechanistic event in prion diseases is the aggregation of aberrantly folded prion protein into large amyloid plaques and fibrous structures associated with neurodegeneration. The cellular prion protein (PrP^C) is absolutely required for disease development, and prion knockout mice are not susceptible to prion disease. Prions accumulate not only in the central nervous system but also in lymphoid organs, as shown for new variant and sporadic Creutzfeldt-Jakob patients and for some animals. To date it is largely accepted that prions consist primarily of PrP^{Sc}, a misfolded and aggregated β-sheet-rich isoform of PrP^C. However, PrP^{Sc} may or may not be completely congruent with the infectious moiety. Here, we discuss the molecular mechanisms leading to neurodegeneration, the role of the immune system in prion pathogenesis, and the existence of prion strains that appear to have different tropisms and biochemical characteristics.

INTRODUCTION

Prion Diseases: A Medical and Economic Crisis

Transmissible spongiform encephalopathies (TSEs), or prion diseases, are neurodegenerative diseases with an inexorably fatal outcome for the affected host. As of today, no therapy other than palliation is available. Prion diseases have been responsible for entire centuries of tragic episodes: From the end of the nineteenth to the middle of the twentieth century, ritualistic anthropophagy of central nervous system (CNS)-derived tissues in Papua New Guinea tribes led to kuru, which became the prime cause of death in some tribes (1). In the last quarter of the twentieth century, iatrogenic transmission of prion-contaminated gonadotropins into humans caused ≥250 victims of Creutzfeldt-Jakob disease (CJD). In the past 20 years, more than 280,000 cattle suffering from bovine spongiform encephalopathy (BSE) (**Figure 1***a*) provoked a worldwide, major food crisis with so far incomparable economic consequences for the European Union and other countries (2). In addition, transmission of BSE to humans is believed to have caused ≥200 cases of variant of Creutzfeldt-Jakob disease (vCJD) (**Figure 1***b*) (3, 4, 5). The fact that most likely millions of people have been in contact with BSE-contaminated meat initiated a widespread health scare.

The good news is that the BSE crisis has been largely resolved: Few cows succumb to this disease annually, at least in those countries that have implemented effective epidemiological screening systems. Furthermore, the incidence of vCJD, which for a while was feared to rise to pandemic proportions, has not shown a significant rise in the number of total cases (**Figure 1***b*). Today the number of vCJD cases is slightly higher in all non–United Kingdom countries than in the United Kingdom itself.

Despite the encouraging news, the challenges posed by prions to human and animal health are far from over. Not only do we lack answers to many basic questions in the prion field, but we are also confronted with novel, emerging problems in the human medical and veterinary realms.

The United States has witnessed an enigmatic rise of chronic wasting disease (CWD) cases affecting elk and deer (7), as well as the occurrence of the first cases of BSE (8). Furthermore, there has been a recrudescence of scrapie outbreaks among European sheep flocks (e.g., Sweden, Austria, Sardinia). The resurgence of new cases might be linked to an increased sensitivity and frequency of the currently executed testing procedures. These data also underline our deficit in knowledge about prion epidemiology and possible transmission routes of prion diseases in humans and animals.

As an example in the field of human medicine, four cases of vCJD have been reported to be caused by blood transfusion (9–11). This indicates that BSE prions can be recycled among humans, which has caused considerable alarm that the supply of blood-derived pharmaceuticals may be threatened (12). In particular, the report of a subclinical blood-derived vCJD infection in an individual carrying a heterozygote methionine/valine polymorphism at codon 129 of the human *PRNP* gene (10) suggests that transmission of BSE prions to humans enhances their virulence and broadens the spectrum of susceptible recipients. In this respect, it has been demonstrated that polymorphisms at codon 129 of the human *PRNP* gene control susceptibility and incubation time in human patients (e.g., 129MM versus 129MV or 129VV drastically increases the susceptibility of humans to BSE prions). It was reported only recently that most individuals who suffered from kuru and were polymorphic at codon 129 showed incubation times longer than 50 years (13).

Moreover, recent reports indicate that there is still a lot to be learned about the mechanisms of prion transmission (e.g., human to human or within scrapie-affected animal flocks) and prion tropism underlying

the complex alternating distribution patterns of PrP^{Sc} (e.g., PrP^{Sc} deposition in lymphoid tissue, the CNS) and prion infectivity under varying conditions (e.g., chronic inflammation) and hosts (e.g., sheep, elk and deer, human): Chronic inflammation can alter the tropism of prion infectivity or PrP^{Sc} to organs hitherto believed prion free (e.g., liver, pancreas, kidney of mice, mammary gland of sheep, muscle of humans) (14–16). Moreover, PrP^{Sc} was reported in spleen and muscle tissue of sporadic Creutzfeldt-Jakob disease (sCJD) patients (17), and prion infectivity was demonstrated in muscle, blood, and saliva of deer suffering from CWD (18, 189). Also, prion infectivity was shown to be excreted via urine of prion-infected nephritic mice, a process defined as prionuria (19).

These results emphasize the need for further assessment of possible public health risks from TSE-affected extraneural organs. It is very well possible that preexisting pathophysiological conditions of the infected host additionally contributed to unexpected distribution patterns of prion infectivity. For example, the presence of prion infectivity in the blood of sheep or deer may influence the deposition of prion infectivity in various organs previously deemed prion free. Therefore, it should be carefully reconsidered whether only organs of the CNS and the lymphoreticular system should be included in the current risk classifications of biologicals in the future. It will be important to test altered prion tropism profiles in nonlymphoid organs and body fluids (e.g., blood, urine, milk, saliva) of ruminants (e.g., sheep, goat, cattle, elk, and deer) and human patients suffering from sCJD and vCJD.

In addition to the eminent questions of the mechanisms of prion transmissions within herds of ruminants, a number of looming questions about the safety of foods and drugs with regard to prion contamination remain unanswered. Moreover, many aspects of the basic biology of prions are essentially unclear. For instance, there is very little understanding of the mechanisms of prion replication at the molecular level. Also, the mechanisms underlying the phenomena of prion strains, prion neurotoxicity, and horizontal prion transmission remain sketchy at best. Diagnostic tools to detect prions with consistent, high sensitivity are still pending; in particular, no test is currently available that can detect prion infectivity in human blood. However, prion science has attracted a vibrant research community that has made scientific and technological inroads in recent years.

For the reasons described above, prion diseases still present a major challenge for biomedical and basic research, representing a fascinating biological phenomenon that has elicited a tremendous interdisciplinary research effort at the interface between neuroscience, structural and molecular biology, and neuroimmunology. The fact that proteins impart their conformational information on other proteins, replicate in the periphery of the infected host, and transmigrate into the CNS, where they induce a fatal neurodegenerative disease, has formed a new dogma. It is very likely that similar posttranslational modifications occur in many different proteins routinely in eukaryotic and prokaryotic cells. These modifications may not always be noxious, and may instead constitute a regulatory process of posttranslational processing influencing function, aggregation status, stability, or subcellular localization of many proteins. Similar phenomena were found for some proteins in yeast and fungi (20).

THE CELLULAR PRION PROTEIN

The cellular prion protein (PrP^{C}) is a glycosyl phosphatidyl inositol (GPI)-linked glycoprotein undergoing facultative N-linked glycosylation at two sites. Like other GPI-linked proteins, it is enriched in detergent-resistant membranes. The structures of mature PrP^{C} from mouse, human, cattle, and Syrian hamster share common features: a long, flexible N-terminal tail (residues 23–128), three α-helices, and a two-stranded antiparallel β-sheet that flanks the first α-helix (**Figure 2a**

and *2b*) (21). The second β-sheet and the third α-helix are connected by a large loop with interesting structural properties. This loop is extremely flexible in most species, but it is almost entirely rigid in the prion protein of elk and deer (22). It remains to be seen whether this structural peculiarity is in any way connected to the propensity of elk and deer to

a

Worldwide reported BSE cases

b

Worldwide reported vCJD cases
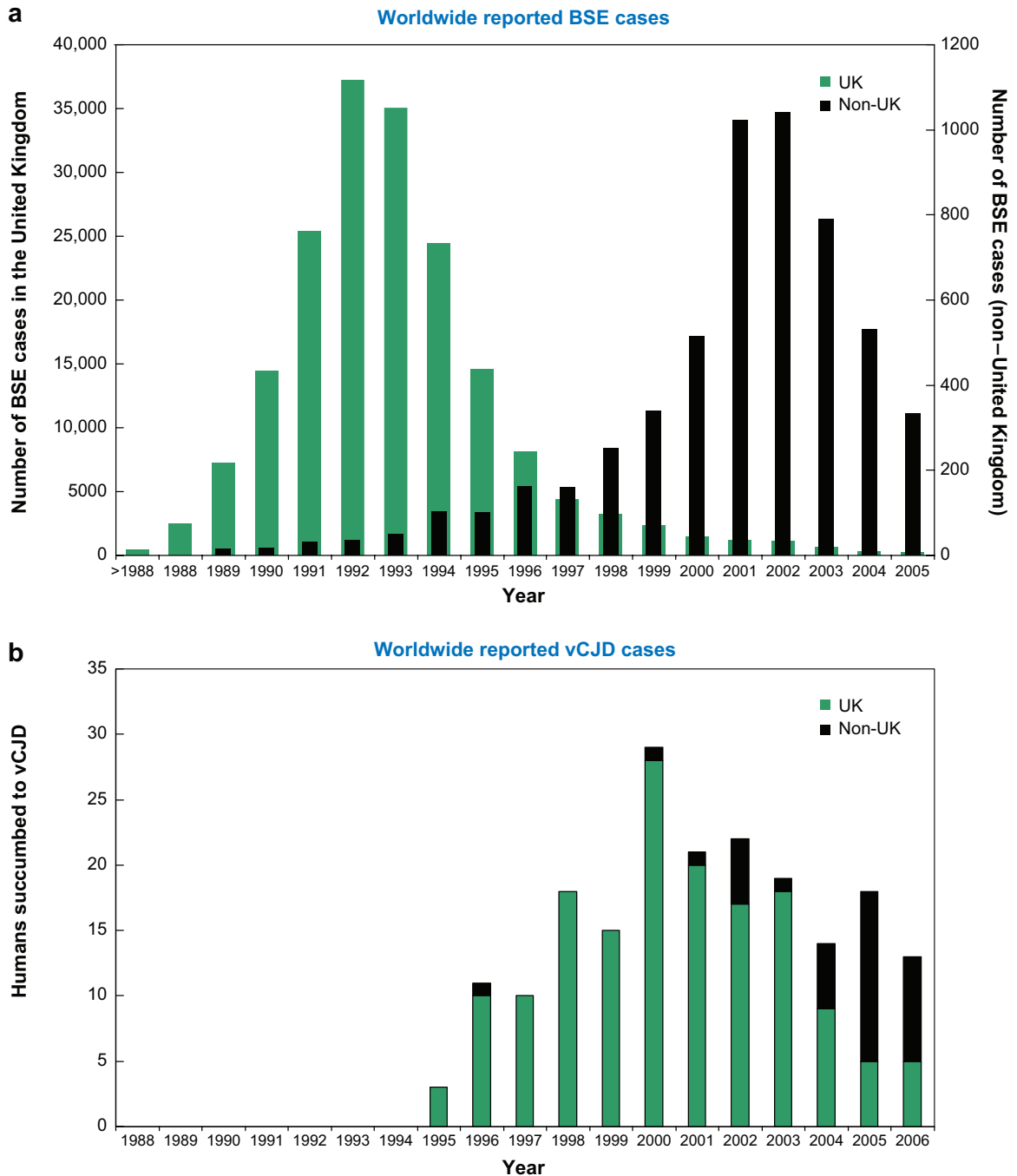
develop CWD. The carboxyl terminus of PrP^C is stabilized by a disulfide bond linking helices two and three (**Figure 2a** and **2b**) (23).

Even if the N-terminal portion of the molecule appears unstructured, it contains two defined, conserved regions. The first consists of a segment with five repeats of an octameric amino acid sequence (octapeptide repeat region) (**Figure 2a**) (21). This region has been proposed to be important in copper binding and might be somehow involved in prion pathogenesis (26). The second region, downstream relative to the first region, contains a highly hydrophobic and conserved profile, which was originally termed transmembrane region 1. However, as it is unclear whether this domain really functions as a transmembrane region under physiological conditions, we propose to rename this region the hydrophobic core domain. It is preceded by a hydrophilic domain termed charge cluster (**Figure 2a**).

PrP^C is a highly conserved protein in mammals, and paralogs are present in turtles (27) and possibly even in amphibians (28). No natural *Prnp*-null alleles have been described in any mammalian species. The broad, diverse, developmentally regulated (29) expression pattern of PrP^C in skeletal muscle, kidney, heart, secondary lymphoid organs, and the CNS suggests a conserved and broad function (30, 31). Within the CNS, high PrP^C expression levels can be detected in synaptic membranes of neurons, but PrP^C is also ex-

pressed in astrocytes (32). In the periphery, PrP^C expression is reported on lymphocytes and at high levels on follicular dendritic cells (FDCs) (30).

WHAT IS THE PHYSIOLOGICAL FUNCTION OF PrP^C?

The *Prnp* gene was identified in 1986 (33) and *Prnp* knockout mice have existed since 1992 (34), yet the function of PrP^C has not been fully clarified. Many recent experiments have focused on elucidating various characteristics of the infectious prion agent, but there have been even more attempts to define the physiological function of the PrP^C. Wüthrich and many others have suggested that the physiological role of PrP^C may help in understanding the pathophysiological properties of prions in general (35). Many different functions have been attributed to PrP^C, including immunoregulation, signal transduction, copper binding, synaptic transmission, induction of apoptosis or protection against apoptotic stimuli, and many others (30). Importantly, postnatal depletion of PrP^C in neurons does not result in neurodegeneration (36). However, neuronal apoptosis in the hippocampus and cerebellum was observed following intracranial delivery of monoclonal PrP antibodies in vivo (37). Only dimerization of PrP^C was shown to induce this phenotype, pointing to the fact that PrP^C dimerization induces an apoptotic signal. Moreover, caspase-12 and endoplasmic reticulum stress were reported

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Figure 1

Incidence of bovine spongiform encephalopathy (BSE) and variant of Creutzfeldt-Jakob disease (vCJD) cases reported worldwide. (*a*) Reported BSE cases in the United Kingdom and in countries and states excluding the United Kingdom. Non–United Kingdom BSE cases include cases from countries of the European Union and outside the European Union (Canada, Israel, Liechtenstein, Japan, Switzerland, and the United States). Data are as of the end of 2005 and also include imported cases (4). (*b*) Reported cases of vCJD in the United Kingdom and in countries outside the United Kingdom. Non–United Kingdom vCJD cases include cases reported in France, the Republic of Ireland, Italy, the United States, Canada, Saudi Arabia, Japan, Netherlands, Portugal, and Spain. Data are as of October 2006, including cases of vCJD patients with a residential history in the United Kingdom in the 1980s and 1990s (5). Note that since 2005, the incidence of vCJD in France has surpassed that of the United Kingdom. Reprinted by permission from Macmillan Publishers Ltd: *Nat. Rev. Mol. Cell. Biol.* 8:552–61, copyright 2007.
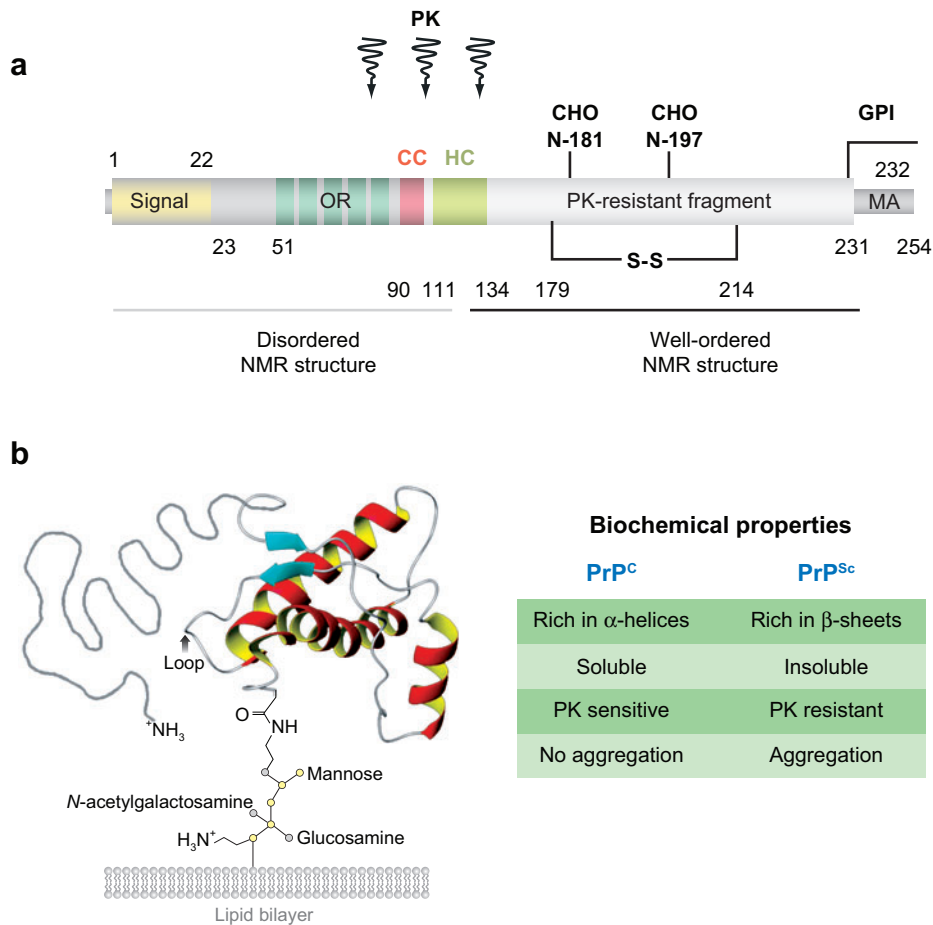
Figure 2

Structural features and biochemical properties of the cellular prion protein. (*a*) Scheme of the primary structure of the cellular prion protein and its posttranslational modifications. A secretory signal peptide resides at the extreme N terminus. The numbers describe the positions of the respective amino acids. The proteinase K (PK)-resistant core of PrP^{Sc} is depicted in gray; the approximate cutting site of PK within PrP^{Sc} is indicated by arrows. CC (*pink*), charged cluster; HC (*green*), hydrophobic core; S-S, single disulfide bridge; MA, membrane anchor region; GPI, glycosyl phosphatidyl inositol; CHO, facultative glycosylation sites; NMR nuclear magnetic resonance. (*b*) Tertiary structure of the cellular prion protein, as deduced from NMR spectroscopy, inserted into a lipid bilayer, including the unstructured N-terminal tail (*gray*) and the GPI anchor. The α-helices are indicated in red; the antiparallel β-sheets are shown in turquoise. Sugar residues are shown as colored small circles. See figure for biochemical properties of PrP^{C} and PrP^{Sc}. Figure adapted with permission from References 24 and 25.

to mediate neurotoxicity of the pathological prion protein in vitro (38).

In addition to the expression of PrP^{C} in the CNS, on circulating (e.g., T and B cells) and resident cells of the immune system (e.g., FDCs), PrP^{C} is also expressed on long-term repopulating hematopoietic stem cells (39).

There it is believed to positively regulate the proliferation of neural precursors during developmental and adult mammalian neurogenesis (40). Whatever the function of PrP^{C} is, upon conversion to PrP^{Sc} it may be altered, and this may constitute a plausible cause of neurodegeneration (35).

A PARTNER FOR PrP^C: DATING THE PROTEOME

In addition to the unknown function of PrP^C, prionologists are occupied with another crucial question: What are the interaction partners of PrP^C? Finding a specific interaction partner of PrP^C might not only be an important step forward in explaining PrP^C function, it might also help explain the role of PrP^C and PrP^{Sc} in the induction of neurodegeneration. Many efforts have been undertaken to find interaction partners of PrP^C and indeed many have been found (N-CAM, laminin receptor, Bax, Bcl-2, etc.) in vitro and in vivo (41–43). However, none of the candidates was so far demonstrated to be of importance in vivo (30, 35), and none of the identified interaction partners has been shown to be implicated in prion pathogenesis. Therefore, one has to conclude that a clear understanding of the physiological function of the PrP^C and its interaction partners is still lacking. The most important discoveries in this respect are therefore still to be made.

MODELS DESCRIBING THE NATURE OF THE PRION PROTEIN AND ITS REPLICATION

The unusual properties of the scrapie agent, such as resistance to UV light, partial resistance to proteinase K (PK), high-pressure treatment, and high temperature, led to speculations that it might consist of protein only (44), or be devoid of both nucleic acid and protein (45), or be a polysaccharide (46) or a membrane fragment (47).

At present, many hypotheses concerning the nature of the scrapie agent have been disproved, and the most commonly discussed hypotheses are outlined here: (*a*) the protein-only hypothesis, (*b*) the virino hypothesis, and (*c*) the hypothesis that stoichiometric transformation of PrP^C to PrP^{Sc} in vitro requires specific RNA molecules.

The protein only hypothesis (44) is currently the most widely accepted model, even though data and scientific opinions do not absolutely conform to this idea (35). As outlined in general terms by Griffith (44), characterized in detailed form by Prusiner (48–51), and refined by Weissmann (52), it suggests that the infectious agent causing TSE is devoid of nucleic acid and is identical to a posttranslationally modified form (PrP^{Sc}) of a host protein (PrP^C). It is possible that it differs from the latter only in the conformational state (53). A large body of epidemiological and experimental evidence is in line with the protein only hypothesis, and very stringently designed experiments have failed to disprove it (30). Knockout mice, carrying a homozygous deletion of the *Prnp* gene that encodes PrP^C, fail to develop disease upon inoculation with infectious brain homogenate (54) and do not carry prion infectivity in the brain (55). Reintroduction of *Prnp* by transgenesis restores infectibility and prion pathogenesis in *Prnp*$^{o/o}$ mice (30). Additionally, all familial cases of human TSEs are characterized by *PRNP* mutations (56). This clearly suggests that PrP^C or mutations thereof are necessary for the development of prion disease. On the basis of this, two different theories explaining the mechanism of PrP^{Sc}-induced conversion of PrP^C to PrP^{Sc} exist: (*a*) the heterodimer, or template-directed refolding, model (57, 58) and (*b*) the noncatalytic nucleated polymerization model (59).

The template-directed refolding model (**Figure 3**) proposes that upon infection of an appropriate host cell, the incoming conformationally altered PrP^C (PrP^{Sc}) starts a catalytic cascade using PrP^C or a partially unfolded intermediate (PrP*) arising from stochastic fluctuations in PrP^C conformations, as a substrate, converting it by a conformational change into a new β-sheet-rich protein (see also **Figure 2*b***). The newly formed PrP^{Sc} will in turn convert a new PrP^C molecule into a new PK-resistant entity. The conformational change is kinetically controlled: A high-activation energy barrier prevents
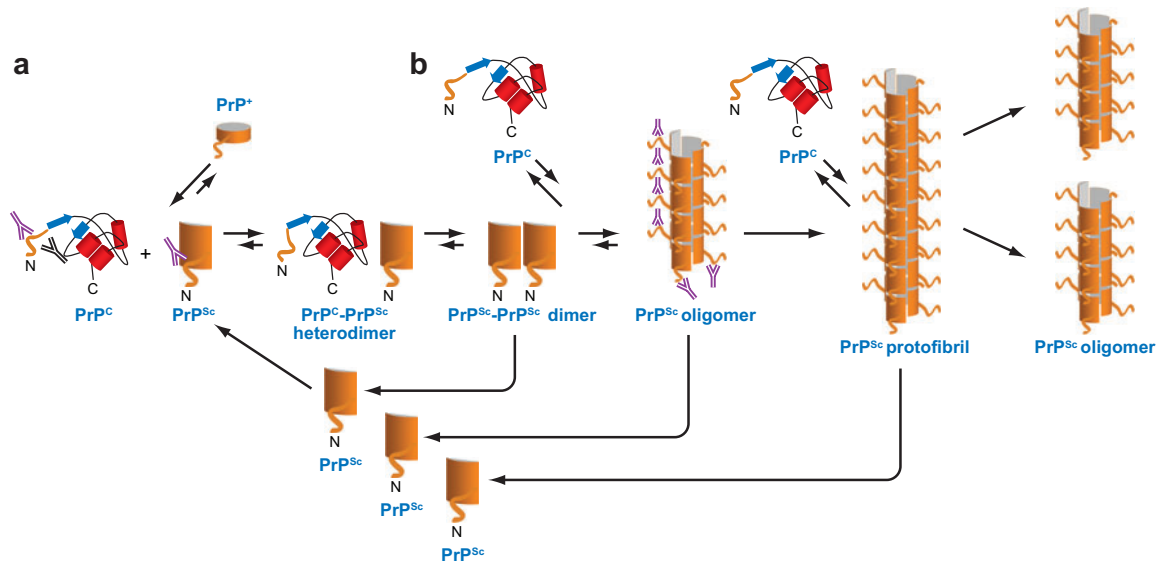
Figure 3

Models of PrP^C to PrP^{Sc} conversion. (*a*) The antibodies POM3 and POM2 (62) are indicated in black and red, respectively. In the case of murine PrP^C and the Rocky Mountain Laboratory prion strain (RML), POM2 binds an epitope on the N terminus of PrP^C (62) that is also present on PrP^{Sc}. In contrast, upon conversion from PrP^C to PrP^{Sc}, the POM3 epitope is buried, and therefore accessible only on the PrP^C molecule. (*b*) The noncatalytic nucleated polymerization model proposes that the conformational change of PrP^C into PrP^{Sc} is thermodynamically controlled: The conversion of PrP^C and PrP^{Sc} is a reversible process, but at equilibrium strongly favors the conformation of PrP^C. Converted PrP^{Sc} is maintained only when it adds onto a fibril-like seed or aggregate of PrP^{Sc}. Once a seed is present, further monomer addition is accelerated.

spontaneous conversion at detectable rates. The formation of a PrP^C into a PrP^{Sc} heteromeric complex (PrP dimer) may lower the activation energy barrier to the formation of new PrP^{Sc} from PrP^C-PrP^{Sc}, leading to further recruitment of PrP^C, which is an autocatalytic process. The extensive unfolding and refolding process is believed to require chaperone activity and energy. Sporadic cases of CJD are thought to be caused by an extremely rare spontaneous conversion of PrP^C to the pathogenic conformation without degradation, with a frequency of about one case per million people per year, as estimated from CJD epidemiology (60). The familial forms of prion disease are tightly linked to certain mutations in the *PRNP* gene (61). Prusiner (61) proposed that these mutations allow spontaneous conversion of PrP^C into PrP^{Sc} with a frequency sufficient to allow the disease to be

expressed within the lifetime of the individual. This increase in probability could be due to lowered activation energy for the transition of the normal to the pathogenic conformation of mutated as compared to wild-type PrP, as proposed above.

The activation energy to switch from PrP^C to PrP^{Sc}, once the initial event has taken place, would be far lower, taking in account the catalytic nature of the further process. Alternatively, the noncatalytic nucleated polymerization model proposes that the conformational change is thermodynamically controlled: The conversion of PrP^C and PrP^{Sc} is a reversible process, but at equilibrium strongly favors the conformation of PrP^C. Converted PrP^{Sc} is established and stabilized only when it adds onto a crystal-like seed or aggregate of PrP^{Sc} (**Figure 3b**). Once a seed is present, further monomer addition is accelerated. According

to this nucleation hypothesis, the aggregated state would be an intrinsic property of infectivity: Monomeric PrP^{Sc} would be harmless, but might be prone to incorporate nascent PrP^{Sc} aggregates (e.g., generating oligomeric PrP^{Sc}).

The final proof for the protein only hypothesis would be, for example, the conversion of noninfectious PrP^{C} into infectious material by biochemical and/or physicochemical interventions as well as by expressing truncated PrP^{C} in vitro (e.g., in eukaryotic or prokaryotic expression systems). In vitro conversion of radioactively labeled PrP^{C} into partially PK-resistant (PrP^{Sc}) material was performed by Bessen, Kocisko, Caughey, and collaborators (63, 64), and brought direct evidence that PrP^{Sc} can induce its own formation. However, the large amounts of the infectious agent used in this assay currently preclude attempts to search for an increase in infectivity (de novo infectivity) in the in vitro conversion products. Moreover, infectivity was never associated with the PK-resistant isoform converted in vitro (65).

In addition, two studies recently provided strong evidence that prions may be synthesized in cell-free systems (66, 67), essentially settling the score as to the nature of the infectious agent. However, the molecular mechanisms of the conversion process—for example, how this is accomplished under physiological conditions (e.g., pH conditions, cofactors) and exactly where it takes place in an in vivo setting (e.g., on the cell surface, in endosomes, in the extracellular space)—remain elusive.

The virino hypothesis (68, 69) postulates that the infectious agent consists of an essential scrapie-specific nucleic acid associated or coated with a host-encoded protein, for which PrP^{C} is the most likely candidate. The host origin of the postulated coat would explain the lack or reduced degree of immunological and inflammatory response, whereas the existence of a nucleic acid would explain how many different strains of scrapie can be propagated in a single inbred mouse line. A tight interaction of nucleic acid genome and PrP^{C} coat, which might cause the infectious particle to be compact and small, could determine the unusual physical resistance of the particle to sterilizing procedures and chemical and biochemical treatments. Despite considerable efforts and the high sensitivity of the tools of modern molecular biology, no evidence for TSE-specific nucleic acids has yet been adduced (30, 70, 71).

Recently it has been reported that stoichiometric transformation of PrP^{C} to PrP^{Sc} in vitro requires specific RNA molecules (72). Notably, whereas mammalian RNA preparations stimulate in vitro amplification of PrP^{Sc}, RNA preparations from invertebrate species do not. These findings suggest that host-encoded stimulatory RNA molecules may have a role in the pathogenesis of prion disease. Nevertheless, it must be stated that all experiments supporting the latter hypothesis were conducted in vitro. Therefore, these results need to be confirmed in vivo (30).

PATHOLOGY OF HUMAN PRION DISEASES

Rapidly progressive dementia, myoclonus, visual or cerebellar signs, pyramidal/extrapyramidal signs, and akinetic mutism clinically characterize fatal neurological diseases caused by prions. The most common human prion disease is CJD, of which there are three subtypes: sporadic, infectious, and familial.

All CJD subtypes have in most cases been successfully transmitted to primates by ingestion or inoculation of brain tissue (30, 73, 74), thus fulfilling one of the main characteristics of TSE diseases. The transmitted/iatrogenic group consists of kuru, iatrogenic CJD, and vCJD. For kuru and iatrogenic CJD, patients were exposed to the TSE agent by contact with brain tissues or contaminated tissue, whereas for vCJD, it is believed that this disease is associated with BSE, on the basis of epidemiological evidence

and biochemical similarities of the prion strains.

Sporadic Creutzfeldt-Jakob Disease

Approximately 85% of all human prion diseases are sporadic forms of CJD. In countries that engage in meticulous surveillance, incidences of 0.4–1.8 cases per million people per year are reported (75). For sCJD, there is no association with a mutant PrP allele, nor is there any epidemiological evidence for exposure to a TSE agent through contact with people or animals infected with TSEs. However, heterozygosity (Met/Val) at PrP codon 129 appears to be associated with a lower risk (76) and/or prolonged incubation time (10, 13). The lack of routine laboratory testing for preclinical diagnosis makes the search for agent sources and other risk factors extremely difficult. At present, the means of acquisition of a TSE agent in these patients remains a mystery. So far, there is no evidence for spontaneous PrP^{Sc} formation in any animal or human TSE. In humans, the peak age incidence of sporadic CJD is 55–60 years. However, if spontaneous misfolding were the primary event, one might expect a continuously increasing incidence with age because more time would allow more opportunity for rare misfolding events.

Kuru

Kuru was and still is a slowly progressing neurodegenerative disease in the eastern highlands of Papua New Guinea, described at the beginning of the last century. It was the first human disease related to scrapie (74) when it was recognized that the lesions in the brains of patients were similar to those observed in scrapie. It was hypothesized that the propagation of kuru through the society occurred by ritual cannibalism and may have originated with the consumption of tissues that belonged to a sporadic CJD sufferer (77, 78). Successful transmission of kuru-affected brains to chimpanzees (74) indicated

an infectious agent as the cause of the disease. The incidence of kuru has decreased to low levels upon cessation of cannibalistic rituals. It has been proposed that variations in the human prion gene (*PRNP*) that protect against prion infection (e.g., heterozygosity at codon 129) have disseminated more efficiently among human populations than nonprotective polymorphisms, suggesting selective pressure. Consequently, prion diseases, now exceedingly rare, may have ravaged human populations in the distant past (79). However, several publications have pointed out that these conclusions may be incorrect (80–82). It was recently described that incubation periods of kuru could be as long as 56 years. *PRNP* analysis showed that most of those patients with kuru were heterozygous at polymorphic codon 129, a genotype associated with extended incubation periods and resistance to prion disease (13).

Iatrogenic Creutzfeldt-Jakob Disease

Iatrogenic CJD has been induced by transplantation of corneal or dural tissue from patients with TSE, or by neurosurgery, using instruments incompletely sterilized following use on TSE patients (30). Moreover, iatrogenic CJD has been detected after inoculation of a growth hormone extracted from pituitary glands pooled from large groups of individuals (83). In these circumstances, the extracts were apparently contaminated with brain tissue from an undiagnosed CJD patient. The incubation period, or latent period (time from exposure to the agent until clinical onset), is long, ranging from two years to greater than 10 years. Interestingly, in disease following corneal or dural transplant or the use of contaminated neurosurgical instruments, the latency is considerably shorter (1–2 years). It is likely that direct introduction of the agent into the brain, as in the latter instances, may account for the clinical differences (e.g., in incubation period and clinical signs) observed.

Houston and colleagues (84) demonstrated that is possible to efficiently transmit prions

via blood transfusion in sheep. This route of potential transmission represents a scenario for inadvertent amplification of the new vCJD agent to humans, which was, until recently, thought to be purely hypothetical. However, recent reports present cases where exactly that may have happened (9–11, 85). The first patient was identified to have developed symptoms of vCJD most likely as a consequence of blood transfusion received from an individual harboring the vCJD agent 3.5 years prior to developing clinical signs of prion disease (9). Six and a half years post blood transfusion, the recipient developed clinical signs of prion disease (9). These findings raised the likely possibility that infection was transfusion transmitted.

Variant Creutzfeldt-Jakob Disease

In 1996, neuropathologists in the United Kingdom described vCJD (3). This disease is not a familial disease associated with mutations in the *PRNP* gene as described below. To date, all primary vCJD cases are found to carry the Met/Met allelotype at PrP codon 129, a common genotype in the Caucasian population. vCJD affects primarily young adults and is clinically characterized as a progressive neuropsychiatric disorder leading to ataxia, dementia, and involuntary movements. vCJD can be distinguished from sCJD, the most common human TSE recognized worldwide for decades, by the early age of onset (vCJD: 19–39 years; sCJD: 55–60 years), longer duration of illness (vCJD: 7.5–22 months; sCJD: 2.5–6.5 months), absence of electroencephalographic changes typically found in CJD, and distinct neuropathological features (86). In vCJD, significant amounts of pathological prion protein (PrP^{Sc}) are detectable in the lymphoid tissues in preclinical disease (87). Of note, PrP^{Sc} is also detectable in lymphoid tissues and extralymphoid tissues of sporadic CJD patients by western blotting and immunohistochemistry (17).

The initial occurrence of these patients in the United Kingdom indicated a possible association with BSE in cattle (3). Subsequent laboratory experiments demonstrated a strong similarity between BSE and vCJD on the basis of similar lesion distributions in mouse brains, PrP^{Sc} glycoform gel banding patterns, and neuropathology after transmission to cynomolgus macaques (*Macaca fascicularis*) (88–90). On the basis of these data, vCJD likely represents a spread of BSE from cattle to humans.

Although millions of Europeans were most likely exposed to BSE, only approximately 200 humans have contracted vCJD in past years. Although most cases have been reported in the United Kingdom, other European and non-European countries including France, Italy, and Canada still have cases of vCJD. Therefore, susceptibility may be controlled by endogenous or exogenous factors.

Since 2001, however, the incidence of vCJD in the United Kingdom appears to be stabilizing (**http://www.cjd.ed.ac.uk/vcjdworld.htm**). It may be too early to draw any far-reaching conclusions on the extent of the CJD epidemic, but each year that passes without any dramatic rise in the number of cases increases the hope that the total number of vCJD victims will be limited (91). The incidence of vCJD in the United Kingdom may already be subsiding (**Figure 1b**) (92).

At present, there is concern that some individuals exposed to BSE might be asymptomatic carriers of the infection (93) and that these people might, in turn, pose a risk of further transmission of the infection to others (e.g., blood transfusions, donors for corneal transplantations). Because of this, there has been an increased need to develop adequate sterilization procedures for surgical instruments, as conventional sterilization procedures do not completely abolish infectivity. Moreover, concern that the blood supply might be contaminated with the vCJD agent, as discussed above, has been widely publicized. Many countries have, therefore, passed rules to diminish the use of blood from donors who might have been exposed to BSE/vCJD

in the United Kingdom during the peak of the BSE epidemic.

Familial Transmissible Spongiform Encephalopathies

Familial TSEs are associated with an autosomal dominant *PRNP* gene alteration (94). This disease accounts for 10%–20% of all TSE cases in humans and includes familial CJD, Gerstmann-Sträussler-Scheinker (GSS) syndrome, and fatal familial insomnia. There is variability in the clinical and pathological findings, the age of onset, and the duration depending upon the particular PrP mutation involved. In addition to point mutations in the *PRNP* gene, insertions in the octapeptide repeat region (**Figure 2**) of the *PRNP* gene have been associated with degenerative brain disease. Clinical disease usually begins at an early age and is of a long duration. GSS syndrome is a rare inherited autosomal dominant disease, which is associated with mutations in the *PRNP* gene (the most common are at codons 102 and 198). GSS syndrome is characterized by chronic progressive ataxia, terminal dementia, a long clinical duration (2–10 years), and multicentric amyloid plaques that can be visualized by antibodies directed against the prion protein. Fatal familial insomnia is an inherited disease, which is characterized by sleep disturbances as well as vegetative and focal neurological signs as a result of thalamic lesions. The clinical phenotype depends upon the D178N point mutation of the *PRNP* gene coupled with a methionine at codon 129 (95).

NEUROTOXICITY, INFECTIVITY, AND PrP^{Sc}

It is clear that prions exert their destructive effects predominantly, if not exclusively, within the CNS. However, the proximal cause of neurotoxicity remains unclear. PrP^C is required for prion replication, as mice devoid of PrP^C are resistant to prions (54). Dimeric PrP^C was found to efficaciously bind PrP^{Sc} (96), suggesting that its conversion is somehow instructed by PrP^C. The first evidence for PrP^C-mediated neurotoxicity was provided by grafting neural tissue overexpressing PrP^C into the brain of PrP-deficient mice (97). After intracerebral inoculation with scrapie prions, grafts accumulated high levels of PrP^{Sc} and infectivity, developing characteristic scrapie histopathology. It was then reported that depletion of endogenous neuronal PrP^C in mice with established prion infection reversed early spongiform changes and banned neuronal loss and progression to clinical disease (98).

PrP^C depletion during the conversion process is unlikely to cause pathology because ablation of PrP^C does not result in scrapie-like symptoms (34). This is corroborated by postnatal PrP^C depletion, which does not result in neurodegeneration (36). However, it could be possible that PrP^C function is altered upon conversion to PrP^{Sc}, leading to neurodegeneration (30, 35). Although neurotoxic, quite surprisingly, high prion titers in lymphoid organs are not accompanied by significant histopathological changes (99, 100), even though murine scrapie infection was recently reported to cause an abnormal germinal center reaction in the spleen (101).

Expression of a PrP variant targeted to the cytosol was found to be toxic to cultured cells and transgenic mice, and it was speculated that this feature might be common to diverse prion-related neurodegenerative disorders (102, 103). Mutant PrP^C, lacking GPI anchor and its signal peptide, retrogradely transported out of the endoplasmatic reticulum induced the generation of amorphous PrP aggregates that possessed partial PK resistance in the cytosol (103). The disease was not reported to be transmissible, which is, after all, the crucial defining trait of a prion disease. Subsequent reports have argued against the contribution of a cytosolic neurotoxic PrP species to prion pathology (104), and therefore this question should be considered unresolved at the present stage.

Transgenic mice expressing N-terminal-deleted variants of PrP were found to suffer from unexpected phenotypes, including

cerebellar granular cell degeneration and leukoencephalopathy (105, 106). Deletions of amino acids 32–121 or 32–134 (collectively termed ΔPrP) confer strong neurotoxicity to PrP^C in vivo, a pathology that can be abrogated by reintroduction of wild-type, full-length PrP^C (105). The latter phenomenon suggests that ΔPrP is a functional antagonist of PrP^C. If so, suppression of ΔPrP toxicity may be used for probing the functional integrity of PrP mutants. This strategy has been used extensively by us and others, as it allows one to map functional domains within the *Prnp* gene, even if the function of PrP is still not understood.

PrP^C contains a highly hydrophobic stretch at the border between its flexible N-terminal and its globular C-terminal part. This particular stretch is believed to play an important functional role, and its manipulation may provide significant functional insights: Recent studies suggest that a small deletion within this hydrophobic stretch (amino acids 121–134) suffices to produce a highly neurotoxic molecule (107). Another neurotoxic type of PrP was reported by Hegde et al. (108), who discovered that the hydrophobic domain acquires a transmembrane localization in a small fraction of PrP molecules in contrast to abundantly GPI-anchored PrP molecules. Expression levels of transmembrane PrP (^{Ctm}PrP) are elevated in certain pathogenic PrP mutants, which are neurotoxic when expressed at high levels in transgenic animals (109). Surprisingly, when co-expressed with full-length PrP, ^{Ctm}PrP is even more neurotoxic. In this regard, it behaves very differently from N-terminally truncated PrP, whose toxicity is reduced or abolished by the expression of full-length PrP.

Although the normal function of PrP is presumed beneficial, there is a growing list of malicious consequences beyond prion diseases that can be elicited by manipulating PrP. Such consequences encompass not only the neurological syndrome (termed Shmerling's disease) elicited by ΔPrP family members.

It was also found that antibody-mediated cross-linking of PrP in vivo triggers neuronal apoptosis in the hippocampus and cerebellum (37). This effect was induced by dimerization of PrP^C through the intracranial stereotaxic delivery of bivalent immunoglobulins. None of the molecular mechanisms underlying these observations have been elucidated, but there has been much speculation that cross-linking may induce cytotoxic lethal signaling cascades. Recently, Chesebro and colleagues generated an interesting *Prnp* transgene that lacks the signal peptide responsible for GPI anchoring (110). Consequently, transgenic mice expressed exclusively a secreted form of PrP^C. Although GPI-negative transgenic mice did not develop clinical disease upon prion infection, their brains contained PrP^{Sc} plaques. Evidently, removal of the GPI anchor abolished the susceptibility to clinical disease while preserving the competence of the soluble PrP^C molecule to support prion replication (110). This observation fits with the growing body of evidence that PrP^C may function as a signaling molecule, just like other GPI-linked proteins (111).

Additionally, the brain, blood, and heart of GPI-negative transgenic mice contained both abnormal protease-resistant prion protein as well as prion infectivity (112). Blood plasma of GPI-negative transgenic mice was found to be infectious (>7 log LD50 infectious units ml^{-1}) (112), mimicking a situation of blood-borne prion infectivity as known from scrapie sick sheep (84), chronic wasting diseased elk and deer (18), and vCJD patients (9, 10). Interestingly, the hearts of these transgenic mice contained PrP^{Sc}-positive amyloid deposits, leading to myocardial stiffness and cardiac disease (112).

Although the exact composition of the infectious prion agent remains elusive, the size of the most infectious moiety was determined (113). The PK-resistant core of PrP^{Sc} was partially disaggregated, fractionated by size, and analyzed by light scattering and nondenaturing gel electrophoresis. Analyses revealed that nonfibrillar particles, with masses equivalent

to 14–28 PrP molecules, are the most infectious particles. These very exciting data suggest that the Ur prion is indeed an oligomeric seed, that is, a small, ordered aggregate that possesses the capability of growing by means of recruiting monomeric PrP further into itself.

Harris and colleagues (114) generated Tg [PrP–enhanced green fluorescent protein (EGFP)] mice, which express an EGFP-tagged version of the prion protein. This fusion protein behaves like endogenous PrP in terms of its posttranslational processing, subcellular localization, and functional activity—as measured by suppression of Shmerling's disease. Although not convertible to PrP^{Sc} when expressed by itself, the fusion protein was incorporated into scrapie fibrils in brains of prion-infected animals. Co-expression of the transgene and wild-type PrP resulted in progressive accumulation of fluorescent PrP-EGFP aggregates in neuropil, axons, and the Golgi apparatus of neurons, upon prion inoculation. These results identified intracellular sites of PrP^{Sc} aggregation that had not been visualized thus far (115), and provided a novel and potentially extremely useful reagent for the study of PrP aggregation.

PRION PATHOGENESIS: LESSONS FROM THE MOUSE MODEL

PrP^C itself is involved in transporting prion infectivity from peripheral sites to the CNS. Adoptive transfer with wild-type bone marrow (BM) into *Prnp*$^{o/o}$ mice reconstitutes the capability of the spleen to accumulate high titers of prion infectivity up to 300 dpi (116, 117). However, reconstitution experiments with wild-type BM into *Prnp*$^{o/o}$ mice were insufficient to restore neuroinvasion. Therefore, hematopoietic cells (e.g., B and T cells, macrophages, and dendritic cells) facilitate the transport of prions from peripheral entry sites to secondary lymphoid organs, in which prions accumulate and/or replicate, although the primary compartment for prion neuroinvasion appears to be nonhematopoietic because it cannot be adoptively transferred by BM reconstitution (116–118).

But how do prions reach the brain following a natural route of exposure, for example, via ingestion? And which cellular and molecular preconditions enable efficient transport? These questions were studied intensively in vitro and in vivo: An in vitro study has shown that microfold cells, specialized intestinal epithelial cells that transfer antigens including pathogens through the epithelium, can transport infectious prions from the apical to the basolateral surface (119). Subsequently, prion neural entry and transit to the CNS may occur with direct prion uptake by nerve endings in the intestine (or spleen after an intraperitoneal exposure) and/or possibly following an amplification phase in the lymphoid tissue; Peyer's patches are required for prion disease development in the mouse (120). Upon interference with lymphoid prion accumulation, many studies have shown disruption and delay in the development of prion disease (121, 122).

Some of the lymphoid players crucial to peripheral prion accumulation have been revealed. Clearly, functional FDCs are essential (121, 123), but it is not entirely clear whether they are the only site of lymphoreticular prion replication or accumulation (116, 117). B lymphocytes are also necessary (118), as they provide maturation signals for FDCs. There is an ill-characterized BM-derived cell population that clearly supports prion replication (117, 124), although this may not apply to all prion strains (125). In addition, morphologic evidence based on time-course studies indicates involvement of the vagal nerve and the sympathetic nervous system as routes of peripheral prion transport to the CNS (126). Glatzel et al. (127) have shown that the sympathetic nervous system is essentially involved in neuroinvasion: Mice with sympathectomy show a significantly prolonged incubation period, and transgenic mice overexpressing the nerve growth factor transgene that have sympathetic hyperinnervation of lymphoid organs

show an accelerated incubation period after a peripheral prion exposure, illustrating the significance of these peripheral nerves in prion pathogenesis.

In the mouse scrapie model, some forms of immunodeficiency impair prion replication and delay disease development, illustrating the significant contribution of this early lymphoid phase. For example, severe combined immunodeficient mice, RAG-1$^{-/-}$, and μMT mice completely resist intraperitoneal prion infection (118). However, replacement of B lymphocyte populations, whether they express PrP^c or not, restores prion infectibility, possibly owing to the key role of mature B lymphocytes in the FDC maturation by provision of tumor necrosis factor and lymphotoxins. PrP^{Sc} heavily decorates FDC membranes in secondary follicles of the spleen (128), lymph node, tonsils, and Peyer's patches in several prion diseases, including vCJD, scrapie, and CWD.

The importance of FDCs in peripheral prion pathogenesis may be exploited for prion prevention strategies. Inhibiting the lymphotoxin beta receptor (LTβR) pathway in mice and nonhuman primates by treatment with LTβR-immunoglobulin fusion protein results in the disappearance of mature, functional FDCs (129, 130). Indeed, treatment with LTβR-immunoglobulin fusion protein was found to impair peripheral prion pathogenesis (121, 123, 131).

The detection of PrP^{Sc} in spleens of sCJD patients (17) suggests that the interface between cells of the immune system and peripheral nerves might also be of relevance in sporadic prion diseases. Indeed, in mouse scrapie studies, there is no doubt that the microarchitecture of lymphoid organs crucially controls the efficacy of prion neuroinvasion: Manipulation of the distance between FDCs and major splenic nerves affects the velocity of neuroinvasion (132). By ablation of the CXCR5 chemokine receptor, lymphocytes were directed toward specific microcompartments, which reduced the distance between germinal-center-associated FDCs and nerve endings (133). This resulted in an increased rate of prion entry into the CNS in CXCR5$^{-/-}$ mice, most likely owing to repositioning of FDCs in juxtaposition with highly innervated, splenic arterioles. It remains to be determined whether the increased rate of neuroinvasion results from a passive diffusion of prions [e.g., released FDC exosomes (134, 135)] or whether mobile cells such as dendritic cells or B cells located in the germinal center are involved in an active process of transport. However, the cells involved in early transport remain unclear. Some evidence for the involvement of dendritic cells has accrued (136). However, other mobile elements, including budding viruses, could also serve as vehicles of infectivity (137).

Because FDCs bind to opsonized antigens via the CD21/CD35 complement receptors, is complement involved in prion pathogenesis? Indeed, mice that lack various complement factors including C1q (138), or that have been depleted of the C3 complement component (139), enjoy enhanced resistance to peripheral prion inoculation. C1q was shown to directly bind PrP in vitro (140). Human studies also point to a possible role for members of the classical complement cascade in prion pathogenesis (141); however, their precise role in prion disease is unknown.

As proinflammatory cytokines and immune cells are involved in lymphoid prion replication (121, 125, 132, 142), we assessed whether chronic inflammatory conditions within nonlymphoid organs could affect the dynamics of prion distribution. Indeed, inclusion body myositis, which is an inflammatory disease of the muscle, was associated with large PrP^{Sc} deposits in muscle (16). Therefore, mice with nephritis, hepatitis, or pancreatitis were inoculated with mouse prions (Rocky Mountain Laboratory strain) and were found to accumulate prions in these otherwise prion-free organs. The presence of inflammatory foci consistently correlated with the upregulation of lymphotoxins α and β (LTs) and the ectopic induction of PrP^C-expressing FDC cells (14).

These data raised concerns that analogous phenomena might occur in TSE-susceptible ruminants. Indeed, we found that sheep with natural scrapie infections and concurrent mastitis harbored PrP^{Sc} in their mammary glands (15), indicating that inflammatory conditions induce accumulation of prions in organs previously believed to be prion free. Therefore, inflammation might indeed be a license for prion replication in nonlymphoid peripheral organs (**Figure 4**), and other organized chronic inflammatory disorders could potentially be sites of prion accumulation and replication.

In addition, it was hypothesized that inflammatory conditions could result in the shedding of prions via excretory organs (e.g., kidney). To investigate this hypothesis, various transgenic and spontaneous mouse models of nephritis were analyzed to ascertain whether prions could be excreted via urine (19). Indeed, prion infectivity was observed in the urine of mice with both subclinical and terminal scrapie, and with inflammatory conditions of the kidney (19).

The genetic or environmental factors that enable horizontal prion spread between hosts have been perplexing. It is possible that the horizontal spread of prions is mediated by secreted body fluids (e.g., urine, milk) derived from potentially infectious secretory organs (e.g., mammary gland, kidney). Placenta of infected ewes could provide a source of prion infectivity for horizontal transmission (143). However, the set of data supporting the latter hypothesis is scant at best.

Public health considerations mandate that we should increase our understanding of the altered prion tropism observed in ruminants (e.g., sheep, cattle, goat, elk, and deer) and the underlying mechanisms. Future experiments should include an analysis of the effect of other common chronic inflammatory disorders (e.g., granulomatous diseases) in prion-infected animals.

Does a chronic subclinical disease or a permanent carrier status occur in ruminants or in humans? Evidence that such a carrier status
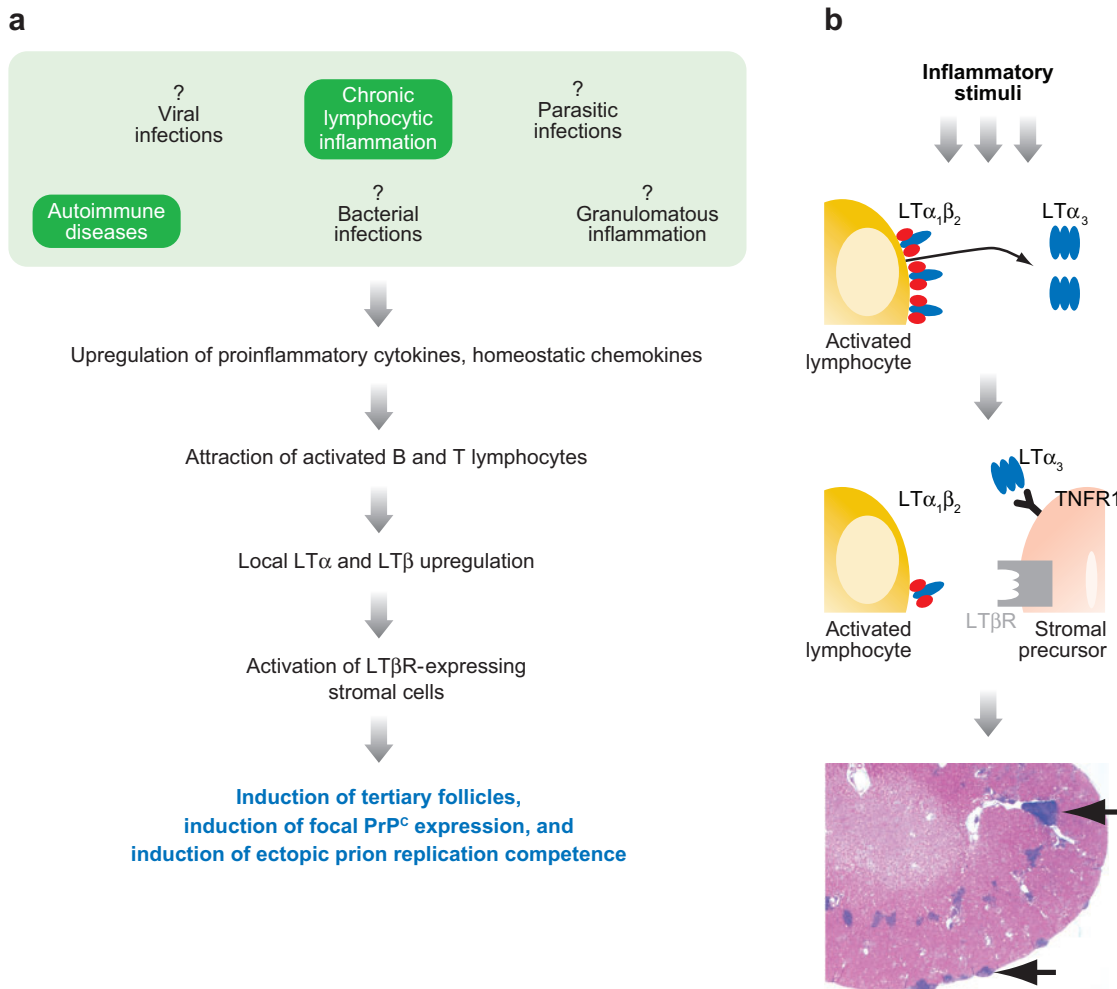
may be produced by the passage of the infectious agent across species was first reported by Race & Chesebro (144) and has been confirmed by others (145, 146), at least for the passage between hamsters and mice. Chesebro reported that mice inoculated with hamster prions lived a long symptom-free life and did not accumulate detectable PrP^{Sc} (35). PrP^{Sc}-negative mouse brains were then injected into naive mice, which had no clinical disease for >650 days. The brains of the latter mice were then passaged to hamsters and resulted in rapid lethality. Therefore, the agent had replicated silently for several years, and could be adoptively transferred, in mice without inducing any clinical signs or histopathology, but maintained full virulence toward hamsters.

Immune deficiency can also lead to a similar situation in which prions replicate silently in the body, even when there is no species barrier (147). So the problem of animal TSEs could be more widespread than is assumed and may call for drastic prion surveillance measures in farm animals, in which healthy animals are tested as well as those with clinical signs of disease. Moreover, people carrying the infectious agent may transmit it horizontally (148), and the risks associated with this possibility can be addressed only if we know more about how the agent is transmitted and how prions reach the brain from peripheral sites.

MECHANISTIC UNDERPINNINGS OF PRION STRAINS

The phenomenon of prion strains has intrigued scientists for decades. Prion strains are distinguishable by stable incubation periods and the pattern of histopathologic lesions within the brain of the same host species. The protein only hypothesis has difficulty explaining the finding that the propagation of different scrapie strains in mice are homozygous in regard to their *Prnp* gene (30, 35): It suggests that an incoming PrP^{Sc} strain can convert the same PrP precursor into a likeness of

a

Viral infections ?

Chronic lymphocytic inflammation

Parasitic infections ?

Autoimmune diseases

Bacterial infections ?

Granulomatous inflammation ?

Upregulation of proinflammatory cytokines, homeostatic chemokines

Attraction of activated B and T lymphocytes

Local LTα and LTβ upregulation

Activation of LTβR-expressing stromal cells

Induction of tertiary follicles, induction of focal PrP^C expression, and induction of ectopic prion replication competence

b

Inflammatory stimuli

$LT\alpha_1\beta_2$

$LT\alpha_3$

Activated lymphocyte

$LT\alpha_3$

$LT\alpha_1\beta_2$ TNFR1

Activated lymphocyte

LTβR   Stromal precursor

Figure 4

Induction of tertiary follicles and prion replication competence in nonlymphoid organs. (*a*) Hypothetical hierarchical cascade inducing the generation of tertiary follicles in nonlymphoid organs and possibly giving rise to ectopic prion replication competence. Autoimmune diseases as well as chronic lymphocytic inflammatory conditions have been demonstrated to induce prion replication competence in nonlymphoid organs (*green*), whereas others have not been investigated (*question mark*). (*b*) Schematic drawing of the events that contribute to the generation of tertiary follicles in response to an inflammatory stimulus that results in the attraction and activation of lymphocytes and the upregulation of lymphotoxins α and β (LTs). LTβ receptor positive (LTβR$^+$) stromal precursor cells, activated upon binding to LTs (LTα$_3$ or LTα$_1$β$_2$) provided by lymphocytes, lead to the expression of homeostatic chemokines and further LTs, generating a positive feedback loop that leads to the formation of tertiary follicles containing follicular dendritic cells and other cells of the immune system. Reprinted by permission from Macmillan Publishers Ltd: *Nat. Rev. Microbiol.* 4:765–75, copyright 2006.

itself, and that this alone can create distinct disease phenotypes, varying in clinical signs, organ tropism, and regions of prion accumulation in the brain.

It is challenging, but maybe not impossible, to reconcile these intriguing data with Prusiner's protein only hypothesis: Epigenetic, posttranslational strain characteristics

of prions appear to dominate over the primary prion protein sequence of the infected host. The diversity of prion strains is largely believed to be due to conformational flexibility of PrP^{Sc}. The host PrP^{Sc} structure is determined by both the PrP^{Sc} conformation in the donor inoculum and limitations imposed by the host primary PrP structure. Circumstantial evidence suggests that strain phenotypes may be encoded within different conformations of PrP^{Sc} with distinct properties, including stability against chaotropic salts and heat (149), the relative prevalence of the main glycosylated moieties, and the size of the PK-digested PrP^{Sc}. The strain-specific differences in the size of PK-digested PrP^{Sc} molecules are thought to result from individual conformations, leading to exposure of distinct cleavage sites. This was first suggested by experiments with transmissible mink encephalopathy (TME), indicating that prion diversity could indeed be conferred by a single protein with varying three-dimensional structures (150). Although great strides have been made toward understanding the molecular origin of strains, the final proof that conformational variants of PrP^{Sc} represent the biological basis of prion strains is still lacking.

Recent in vivo evidence indicated that a similar phenomenon of conformational variants may occur in Alzheimer's disease (151). Here the existence of Aβ strains that can seed and accelerate aggregation and Aβ pathology was posited. These intriguing observations support the hypothesis that the pathogenetic mechanisms operating in Alzheimer's disease and in prion diseases have more in common than we often appreciate (152). Perhaps future studies will address whether different Aβ strains with distinct biochemical or neuropathological characteristics occur in humans. Can multiple prion strains coexist and effect prion replication? Two subtypes of sporadic CJD have recently been demonstrated to coexist in humans (62). Experimental studies have shown that when two strains infect the same host, one strain can impede the abil-

ity of the second strain to cause disease (153). Bartz and colleagues (154) recently suggested that this might be caused by the suppression of prion replication of the second strain. Strain features are useful for tracing prion infections between species. When transmitted to primates, BSE causes lesions strikingly similar to that of vCJD (155, 156). BSE is most likely transmissible to humans too, and strong circumstantial evidence (157–159) suggests that BSE is the cause of vCJD, which has claimed more than 200 lives in the United Kingdom (3, 160), as well as a much smaller number in some other countries (161).

NATURAL TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES: WHAT IS NEW?

Cattle Prions

More than a few surprises have come from further investigations of prion strains in field cases of TSEs. Until recently, BSE was believed to be associated with one single prion strain, classified by an exclusive and remarkably stable biochemical profile of PrP^{Sc}. However, distinct molecular signatures have recently been discovered through the large-scale screening of cattle mandated by European authorities in the context of BSE surveillance. These atypical profiles fall into either of two groups: H-type cases of protease-resistant fragments with a molecular weight higher than BSE, and bovine amyloidotic spongiform encephalopathy, or L type (lower) (162). To test whether these different biochemical and histopathological properties correspond to distinct strains, the Laude laboratory transmitted H-type-PrP^{Sc} isolates from French cattle into transgenic mice expressing bovine or ovine PrP (163). The recipient mice developed neurological signs exhibiting strain-specific features clearly distinct from that of the classical BSE agent, providing pivotal evidence that the underlying strains are distinct.

Atypical Sheep Scrapie

In 1998, aberrant cases of sheep scrapie were described in Norway and the strain was newly classified as Nor98 (164). Active European Union surveillance later revealed additional cases of atypical scrapie in several other countries (165, 166). Sheep infected with Nor98, or atypical scrapie, accumulated PrP^{Sc} primarily in the cerebellum and cerebral cortex rather than in the brainstem target in the classical strain (167). Additionally, on western blot analysis of atypical scrapie cases, an additional small-molecular-weight (10–12 kDa) PrP fragment appeared after PK digestion and was shown by epitope mapping to lack both N and C termini of PrP (167, 168). Furthermore, atypical scrapie cases occurred not only in the classical scrapie-susceptible genotypes (A136 R154 Q171), but also in genotypes associated with high resistance to classical scrapie (A136 R154 R171) (165, 166). Were these atypical scrapie cases also infectious? In 2001, atypical scrapie cases were shown to be transmissible prion diseases after inoculated ovine PrP-expressing transgenic mice developed disease and prion aggregates (169). In the meantime, several countries appear to be reporting extremely high incidences of atypical scrapie, and in fact atypical scrapie appears to be the rule rather than the exception in some geographical areas.

Chronic Wasting Disease

Among all animal prion diseases, CWD of cervids is likely the most efficiently transmitted. CWD infections occur in mule deer (*Odocoileus hemionus*), white-tailed deer (*O. virginianus*), Rocky Mountain elk (*Cervus elaphus nelsoni*) (170), and moose (*Alces alces shirasi*) (171). Prevalence can reach as high as 30% in dense, free-ranging deer populations and nearly 100% in captive animals (171). Hypotheses for CWD transmission range from spread via direct contact to exposure through grazing in areas contaminated by prion-infected secretions, excretions (saliva, urine, feces), tissues (placenta), or decom-

posed carcasses. Insightful experimental studies have recently revealed two key findings: (*a*) Saliva from CWD-infected deer can transmit disease (18), and (*b*) CWD-infected carcasses allowed to decay naturally in confined pastures can lead to CWD infections in captive deer (172). Additionally, the abundant CWD-prion accumulation within lymphoid tissues may also lead to CWD prion buildup in nonlymphoid organs with lymphoid follicles, as was recently shown in kidney, potentially shifting shedding routes (173). It is unknown whether other types of inflammation, such as the granulomatous inflammation in the intestine seen in Johne's disease (*Mycobacterium avium* subsp. *paratuberculosis*; affects ruminants, including deer and elk) or parasitic inflammation, could lead to or perhaps increase prion excretion by fecal routes. The environmental prion contamination in CWD underscores the difficulties of CWD disease management. Within North America, CWD-infected deer and elk have been detected in 14 states and two Canadian provinces (170, 174, 175).

CWD surveillance in Europe has been more limited. However, in Germany, a total of 7300 captive and free-ranging cervids were tested for CWD with no sign of infection (176). Reindeer or caribou (*Rangifer tarandus*), from North America or Northern Europe respectively, have a highly homologous prion sequence compared with mule deer and thus are likely susceptible to CWD. Other European cervids such as moose and red deer *(C. elaphus)* are also expected to be CWD susceptible.

The deer and elk primary protein structures are highly conserved, as seen in other mammals. Interestingly, a polymorphism at codon 225S/F may influence CWD susceptibility in mule deer. When comparing the frequency of genotypes among CWD-negative and -positive deer ($n = 1482$), the odds that a CWD-infected animal was 225SS was 30 times greater when compared with 225SF, whereas the frequency of 225SF/FF genotypes in CWD-negative deer was 9.3%, but only 0.3% in CWD-positive deer (177).

Additionally, elk have a polymorphism at codon 132 (M/L) of *Prnp*, corresponding to polymorphic codon 129 (M/V) in humans. Elk expressing 132ML and 132LL *Prnp* were reported to be overrepresented among elk with CWD when compared with uninfected controls (178), and 132LL elk experimentally infected with CWD have resisted infection for at least four years, whereas 132MM or 132ML elk ($n = 2$ each) developed terminal clinical prion disease by 23 or 40 months post inoculation, respectively, confirmed by immunohistochemistry and western blotting for PrP^{Sc} (179). White-tailed deer also have *Prnp* polymorphisms that may affect their CWD susceptibility. A reduced susceptibility to CWD was linked to a G96S and a Q95H polymorphism in a study comparing allelic frequencies from CWD-positive and CWD-negative free-ranging Wisconsin white-tailed deer (180).

PRION STRAINS AND ORGAN TROPISM

Definitions of prion strains ease nomenclature and classification but certainly do not explain why distinct strains have different organ tropisms. Indeed, some prion strains favor the CNS as a primary target organ but show low abundance or absence in secondary lymphoid organs (e.g., classical BSE, with gut-associated lymphoid tissue being PrP^{Sc} positive over a transient period of time). In contrast, other prion strains can be detected in the CNS but, in addition, exist to high extent in secondary lymphoid organs (e.g., vCJD, CWD, scrapie) (181). What defines the tropism of a prion strain? One might speculate that tropism is defined by the tertiary and quarternary structure of prions leading to binding or interaction with different molecules (e.g., receptors) and therefore different cells. It may also be possible that cellular binding and uptake of prions remains unaltered, but that efficient conversion is restricted to those particular cells that contain a cofactor compatible with the respective strain. This would necessitate

that cofactors supporting prion conversion of distinct strains exist in a particular cell.

Bartz and colleagues (182) analyzed the role of prion infection of lymphoid tissues in neuroinvasion following oral and intraperitoneal (i.p.) inoculation with the hyper (HY) and drowsy (DY) prion strains of TME. DY TME agent infectivity was not detected in spleen or lymph nodes following i.p. or oral inoculation. Moreover, no clinical disease was observed following i.p. inoculation. In contrast, inoculation of the HY TME agent by the i.p. and oral route resulted in splenic and nodal prion replication, inducing clinical scrapie. To clarify the role of the lymphoid tissue in neuroinvasion, the HY and DY TME agents were inoculated into the tongue, which is highly innervated and commonly shows lesions in ruminants. Following intratongue inoculation, the DY TME agent induced prion disease, with deposits both in the tongue and brainstem nuclei that innervate the tongue. No PrP^{Sc} was found in the spleen or lymph nodes. These data support the hypothesis that the DY TME agent can spread from the tongue to the brain along cranial nerves without requiring agent replication in the lymphoid tissue.

A major challenge in studying various prion strains from cattle, sheep, goats, or humans is to find the appropriate, sensitive recipient bioassay, in which the respective strain of interest or even various strains can be propagated. In most cases, prion transmission of distinct species (e.g., human prions into hamster) is restricted by the species barrier, preventing the characterization of, for example, human or ovine prion strains in mouse models. Therefore, prionologists expressed PrP^{C} proteins of various species in transgenic mice to enable transmission or adaptation experiments (183, 184). This worked very well in many instances of autologous PrP expression, for example, ovine or human PrP (185, 186). Furthermore, reduction of species barrier was shown to be facilitated by high expression levels of heterologous PrP (7). A recent study by the Agrimi group identified an appropriate

rodent model, the bank vole (*Clethrionomys glareolus*), for efficient primary prion transmission of CJD isolates (187). Voles infected with genetic and sCJD isolates reproduced strain-specific neuropathology and accumulated PrP^{Sc} with biochemical properties similar to the human counterpart. Adaptation of genetic CJD isolates to voles showed little or no evidence of a transmission barrier, in contrast to the striking barriers observed during the transmission of mouse, hamster, and sheep prions to voles. This is highly interesting because, although low prion protein sequence homology between man and vole was detected, transmission efficiency was comparable to that reported in transgenic mice carrying a human prion protein (188). Further experiments in this direction could increase our understanding in how sequence and three-dimensional structure and maybe cofactors control species barrier or support the propagation of distinct strains.

FUTURE DIRECTIONS

As discussed in this work, a lot of progress has been accomplished in the identification of prion strains as well as in the understanding of the neurotoxic mechanisms of prions. Although this is extremely encouraging, the molecular basis for neurodegenerative processes observed in prion diseases is poorly understood. The development and appropriate use of new tools and technologies enabled prionologists in recent years to answer a couple long-standing key questions in the field. For example, analysis of prion aggregates by light scattering and nondenaturing gels defined the size of the most infectious prion particle (113).

So what are the open questions of the prion field? Some of the most important issues in the field are to understand how neurotoxicity is induced by the prion agent and why it is not toxic to cells of the immune system. Another emerging aim is to understand prion conversion mechanisms and how strain information is maintained and transmitted. What are the mechanisms that define the tropisms of prion strains? What is the physiological function of PrP^{C}? And finally, the holy grail of all questions in the prion field, what is the exact nature of the prion agent? The ability to answer these questions in the future will rely mainly on the quality of the tools and technologies available to the prion field. As the last years have shown, prionology, a field of interdisciplinary research attracting scientists from many different fields, will move ahead to resolve some of the most important questions of the prion riddle.

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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Errata

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