Current Topics

Prion Diseases and Their Biochemical Mechanisms[†]

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Received January 23, 2009; Revised Manuscript Received February 24, 2009

ABSTRACT: Prion diseases, also known as the transmissible spongiform encephalopathies (TSEs), are a group of fatal neurodegenerative disorders that affect humans and animals. These diseases are intimately associated with conformational conversion of the cellular prion protein, PrP^{C}, into an oligomeric β-sheet-rich form, PrP^{Sc}. A growing number of observations support the once heretical hypothesis that transmission of TSE diseases does not require nucleic acids and that PrP^{Sc} alone can act as an infectious agent. The view that misfolded proteins can be infectious is also supported by recent findings regarding prion phenomena in yeast and other fungi. One of the most intriguing facets of prions is their ability to form different strains, leading to distinct phenotypes of TSE diseases. Within the context of the "protein-only" model, prion strains are believed to be encoded in distinct conformations of misfolded prion protein aggregates. In this review, we describe recent advances in biochemical aspects of prion research, with a special focus on the mechanism of conversion of prion protein to the pathogenic form(s), the emerging structural knowledge of fungal and mammalian prions, and our rapidly growing understanding of the molecular basis of prion strains and their relation to barriers of interspecies transmissibility.

Though the "scrapie agent" was first demonstrated to show infectivity between sheep many years ago, the transmissible spongiform encephalopathies (TSEs)[1] have remained among the most puzzling and enigmatic diseases. The TSEs make up a class of fatal mammalian neurodegenerative disorders that encompass kuru, Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome, and fatal familial insomnia in humans, bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep, and chronic wasting disease in cervids (*1*−*7*). Affecting approximately one in every 1 million people, these rare disorders may arise either spontaneously, via inheritance of a predisposing mutation, or by way of infection. Acquisition of disease by this latter mechanism has drawn intense interest among both scientists and the general public, especially in light of the recent outbreak of BSE ("mad cow" disease) in the United Kingdom (*8*) and indications hat BSE has likely been transmitted to humans, resulting in a new variant of CJD (*2, 4*).

After a protracted incubation period, those affected by these crushing neurodegenerative diseases display clinical symptoms of progressive motor dysfunction, cognitive impairment, and cerebral ataxia. The brains of diseased individuals are highly abnormal, showing characteristic spongiform degeneration, astrogliosis, and accumulation of misfolded protein deposits (*2, 4, 5*). Despite these common characteristics, incubation time and pathology are known to vary considerably among the many prion disorders, and these distinct phenotypes (or "strains" of the TSE agent) appear to faithfully propagate even after repeated passaging in experimental animals (*2, 4, 6*). The transmissibility, long incubation times, and existence of phenotypically distinct TSE strains originally led many to believe that these disorders were caused by a "slow virus". However, despite an intense search, no conclusive evidence to support such a claim has ever been found. The infectious pathogen was later proposed to be proteinaceous in nature due to its unusual resistance to radiation and nuclease activity, treatments which would inactivate a virus or naked nucleic acid (*1, 2*). The term "prion" (or proteinaceous infectious particle) was coined by Stanley Prusiner to describe such a unique pathogen (*1*).

Most researchers currently accept this "protein-only" model, according to which the transmissible pathogen is a misfolded form of the normal cellular prion protein (PrP^{C}) (*1*−*7*). The rouge conformer, PrP^{Sc}, is believed to propagate by binding to PrP^{C} and acting as a template to coerce its refolding into the abnormal PrP^{Sc} isoform. Though ultimate proof that the TSE agent is strictly proteinaceous in nature is still missing, the past few decades have ushered in a wealth of data in support of this model. One of the earliest milestones was the identification and cloning of the

[†] This work was supported by National Institutes of Health Grants N5038604, N5044158, and AG014359.

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[1] Abbreviations: TSE, transmissible spongiform encephalopathy; CJD, Creutzfeldt-Jakob disease; BSE, bovine spongiform encephalopathy; PrP, prion protein; PrP^{C}, cellular prion protein; PrP^{Sc}, disease-associated, proteinase K-resistant prion protein isoform; GPI, glycophosphatidylinositol; rPrP, recombinant prion protein; PK, proteinase K; GALT, gut-associated lymphoid tissue; PMCA, protein misfolding cyclic amplification.

10.1021/bi900108v CCC: $40.75
Published on Web 02/24/2009

normal cellular gene encoding the TSE-associated prion protein (*9*). Remarkably, all known familial human prion diseases have since been shown to segregate with specific mutations within this gene (*2, 4*). The critical link between prion protein and TSE pathogenesis was more firmly established in a landmark study by Büeler and co-workers which demonstrated that PrP-deficient mice are resistant to challenge by the scrapie agent (*10*). Furthermore, an intriguing related development was the finding that prion phenomena exist in yeast and other fungi (*11*−*13*). Studies in these microorganisms offered a powerful experimental model, providing a conclusive "proof of principle" that proteins alone can act as self-propagating infectious agents. In more recent years, in vitro techniques for the generation of infectious PrP aggregates have shown continued improvement, and experimental results come ever closer to conclusive evidence for the protein-only hypothesis of TSE diseases (*14*−*16*). However, despite these and other important advances, many aspects of prion biology, biochemistry, and biophysics remain unclear. This review will address some of these issues, with a special focus on the mechanism of prion protein conversion, our emerging structural knowledge of PrP^{Sc}-like aggregates, as well as the molecular basis of prion strains and their relation to the species barrier.

PRP, A PROTEINACEOUS JEKYLL AND HYDE

Mature PrP^{C} is an ~210-amino acid protein which largely localizes to detergent-resistant subdomains known as "lipid rafts" on the outer surface of the plasma membrane via a C-terminal glycophosphatidylinositol (GPI) anchor (*2*−*6*). High-resolution NMR studies of bacterially expressed recombinant prion protein (rPrP), a model for PrP^{C} lacking any post-translational modifications, have revealed a folded C-terminal domain and an N-terminal region which is largely unstructured (*17*). Depending on the species, the flexible N-terminus contains at least four glycine-rich octapeptide repeats which display a particular affinity for Cu^{2+} (*18*), with reports of weaker binding to other divalent cations such as Zn^{2+}, Fe^{2+}, Ni^{2+}, and Mn^{2+} (*19*). The PrP globular domain is highly conserved over many different species, consisting of two short β-strands and three α-helicies, with a disulfide bond bridging helicies 2 and 3. This domain also contains two potential sites for N-linked glycosylation (*17*).

Despite many intriguing leads, the normal cellular function of PrP^{C} has remained shrouded in mystery. PrP-deficient mice exhibit only subtle phenotypic maladies (*10*), and the proposed physiological roles of PrP^{C} are many, ranging from copper internalization and homeostasis to antiapoptotic activities, protection against oxidative stress, cell adhesion, cell signaling, and the modulation of synaptic structure and function (for a review of potential PrP^{C} function, see refs *20*−*22*). The list of putative PrP^{C} binding partners is equally long; some of these cellular cofactors have been suggested to contribute not only to normal PrP^{C} function but also to the conformational conversion process (see below and refs *20*−*22*).

By what means does this transformation from a seemingly innocuous cellular protein into a monstrous pathogenic isoform(s) occur? Central to the protein-only hypothesis is the idea that replication of prions occurs by self-propagation of infectious PrP^{Sc} via recruitment of normal cellular PrP^{C} (*1*−*7*). Although PrP^{C} and PrP^{Sc} appear to share the same covalent structure, they differ profoundly in biochemical and biophysical properties. Cellular PrP^{C} is monomeric, proteinase-sensitive, and soluble in nonionic detergents, whereas PrP^{Sc} is insoluble and aggregate in nature, showing partial resistance to proteinase K (PK) digestion (*2*−*7*). Consistent with NMR structural data for recombinant prion protein, PrP^{C} isolated from normal brain is primarily α-helical, whereas low-resolution optical spectroscopic measurements reveal that PrP^{Sc} isolated from diseased brain contains mostly β-sheet structure (*2*−*4*). This suggests that the differences in biophysical properties between these two isoforms result from distinct protein conformations. The concept that a single protein can exist in multiple thermodynamically stable conformations would appear to challenge the Anfinsen principle that the three-dimensional structure of a protein is encoded entirely by its amino acid sequence. However, the $PrP^{C} \rightarrow PrP^{Sc}$ conformational conversion does not necessarily violate this maxim, since the process appears to be ultimately associated with polymerization of PrP (*23*), which would be expected to modulate the lowest-free energy conformation of monomeric subunits.

Prion diseases are often associated with the deposition of amyloid-like fibrils similar to those observed in other neurodegenerative disorders such as Alzheimer's, Huntington's, and Parkinson's diseases (*24, 25*). While the proteins forming such aggregates are diverse and vary by disease, all amyloids display resistance to proteolytic digestion, insolubility, and affinity for aromatic dyes such as thioflavin T and Congo red. Furthermore, X-ray fiber diffraction studies of these highly ordered aggregates reveal a common "cross-β" core, where β-strands lay perpendicular and hydrogen bonds parallel to the long fibril axis (*25, 26*). In the case of the β-sheet-rich PrP^{Sc}, any such amyloid-like structure would reside within C-terminal residues ~90−231, a region corresponding to the ~27−30 kDa protease resistant fragment (~17 kDa for unglycosylated PrP) found upon digestion of infected brain homogenate with PK (*2*−*4*).

WHAT MAKES A PRION? LESSONS FROM UNLIKELY SOURCES

Prions in Yeast and Other Fungi. While the term prion was originally coined to distinguish the protein-based infectious pathogen associated with TSE diseases from conventional pathogens such as viruses or viroids, recent observations have since expanded this definition. The inheritable yeast phenotype [*URE3*] was first proposed by Reed Wickner to be the result of a prion-like, self-propagating change in the conformation of a protein involved in nitrogen metabolism, Ure2 (*11*). In later years, amyloid formation of other chromosomally encoded yeast and fungal proteins, such as Sup35, Rnq1, HET-s, and, most recently, Swi1 (*11*−*13, 27*), has been shown to result in adoption of distinct and inheritable "prion states". Struggles to unravel the molecular details of mammalian prion biology have shifted great attention to the study of these prion phenomena in the humble but experimentally more tractable microorganisms of yeast and other fungi. While the precise molecular nature of the infectious TSE agent in mammals is shrouded in mystery, prion states in these simple organisms have been unequivocally shown

to result from aggregation of certain cellular proteins into self-propagating amyloid fibrils (*11*−*13*), where transmission occurs naturally through cell division. Importantly, introduction of amyloid fibrils formed in vitro from bacterially expressed yeast prion proteins into living cells was demonstrated to result in the same inheritable prion states, providing conclusive evidence that proteins alone can act as self-propagating infectious agents (*28, 29*). Remarkably, yeast prions also recapitulate some of the most puzzling properties of their mammalian counterparts, displaying both strain variability and barriers to interspecies transmissibility (*11*−*13*).

Prion proteins in yeast and other fungi share no sequence similarity with their mammalian counterparts. Unlike the region of PrP required for transmission of TSEs, the prion domains of these proteins display, with the exception of HET-s, an exceptionally high content (~40%) of two amyloidogenic amino acids, glutamine and asparagine (*11*−*13*). While PrP aggregation is associated with a pathogenic process, formation of fungal prions results in a phenotypic change which varies with the identity of the aggregating protein. For instance, the best characterized yeast prion state is associated with Sup35, a protein normally involved in translation termination machinery. Conformational conversion of this protein into amyloid fibrils depletes active Sup35 monomer from the cell, and the resultant [*PSI*$^+$] phenotype is defective in protein synthesis (*11*−*13*). Not all fungal prions, however, are deleterious. For example, the prion state [Het-s] of *Podospora anserina*, resulting from formation of amyloid fibrils by the protein HET-s, appears to serve a more functional role. Here, the [Het-s] state has been shown to mediate heterokaryon compatibility, preventing cytoplasmic mixing and exchange of nuclei between incompatible cell genotypes (*30*).

In general, a stable prion state requires some self-propagating protein fold, where amplification of this fold occurs through template-mediated aggregate growth that must exceed the rate of biological clearance. Studies in yeast have revealed that prion amplification is accelerated by amyloid fragmentation, which generates additional ends for template-mediated conformational conversion (*31*). Here, fragmentation is accomplished by the molecular chaperone Hsp104, which is responsible for disaggregating large protein deposits and appears to be required for maintenance of the prion state (*11*−*13*). In the case of Sup35 prions, susceptibility to chaperone-mediated fragmentation in vivo also appears to correlate with distinct [*PSI*$^+$] strains, where Sup35 aggregates showing increased resistance to Hsp104 activity result in a less severe phenotype (*31*). It is unclear, however, whether similar mechanisms exist for the TSEs, given that mammals possess no known homologue to the disaggregating chaperone Hsp104.

Other Infectious Amyloids? Are the infectious properties of prions unique among amyloids? Intriguingly, recent data indicate that at least some amyloid-related disorders, once thought to be noninfectious, may also be transmissible under appropriate circumstances. For example, cerebral injection of brain homogenates from Alzheimer's patients into transgenic mice expressing a mutant amyloid precursor protein was shown to result in neuronal amyloid deposition and neurodegeneration within several months (*32*). However, since control (untreated) animals used in these studies develop a similar disease pathology later in life, it is difficult to conclusively determine whether the diseased brain homogenate used in this study acts as a bona fide infectious agent or simply accelerates a predetermined clinical end point. Perhaps even more intriguing are reports pointing to potential transmissibility of systemic amyloidoses in animals. In the case of mouse senile amyloidosis, marked apolipoprotein A-II amyloid deposition has been observed following either injection or oral ingestion of isolated apolipoprotein A-II fibrils (*33*). Amyloid found in fecal matter and milk of these infected animals has also been demonstrated to induce amyloidosis, providing evidence for more natural routes of transmission (*33, 34*). For serum amyloid protein A, recent data suggest that amyloidosis may be transmitted from one species to another, where amyloid present in the feces of captive cheetahs was used to accelerate amyloidosis in mice treated with an inflammatory stimulus (*35*). While these studies have offered an intriguing glimpse into the relationship between amyloid and infection, it remains unclear whether prion-like transmission is a common property of all amyloid disorders under the appropriate experimental conditions.

PRION NEUROINVASION AND TOXICITY

Although most cases of TSEs arise spontaneously, it is the infectious contraction of disease which has attracted the greatest scientific and public interest. While in the laboratory setting TSE agent is typically delivered by intracerebral inoculation into experimental animals, the most common mechanism for natural spread of the disease is through ingestion. For example, strong evidence suggests that the feeding of BSE-contaminated meat and bonemeal to livestock was responsible for the recent outbreak of BSE in England, and subsequent consumption of diseased cattle by humans is believed to be responsible for the emergence of variant CJD (vCJD) (*2, 4, 5, 8*). Thus, neuroinvasion typically begins upon ingestion of the TSE agent.

The pathogen must first cross the intestinal epithelium in a process that remains unclear, although experimental data point toward a mechanism involving transcytosis by microfold (M) cells (*7, 36*) as shown in Figure 1A. Migratory dendritic cells are also known to directly capture antigens within the intestinal lumen and could also be responsible for initial uptake of the TSE agent. Once past the epithelial wall, PrP^{Sc} appears to be phagocytosed by antigen-displaying cells such as macrophages and dendritic cells. While macrophages appear to serve a more protective role (*7*), some experimental evidence suggests that dendritic cells deliver the TSE agent to follicular dendritic cells located in the germinal centers of B cell-rich follicles present in Peyer's patches and other gut-associated lymphoid tissue (GALT) underlying the intestinal epithelium (Figure 1A). After incubation in lymphoid tissue such as the GALT and spleen, the TSE agent spreads to the central nervous system (CNS) via the enteric nervous system. This invasion occurs in the retrograde direction along efferent fibers of both sympathetic (e.g., the splanchnic nerve) and parasympathetic (e.g., the vagus nerve) nerves (*7, 36*) (Figure 1B). In is unknown how this retrograde transport between synaptically linked peripheral nerve cells occurs: by stepwise interactions involving direct contact between PrP^{Sc} and surface PrP^{C} along the

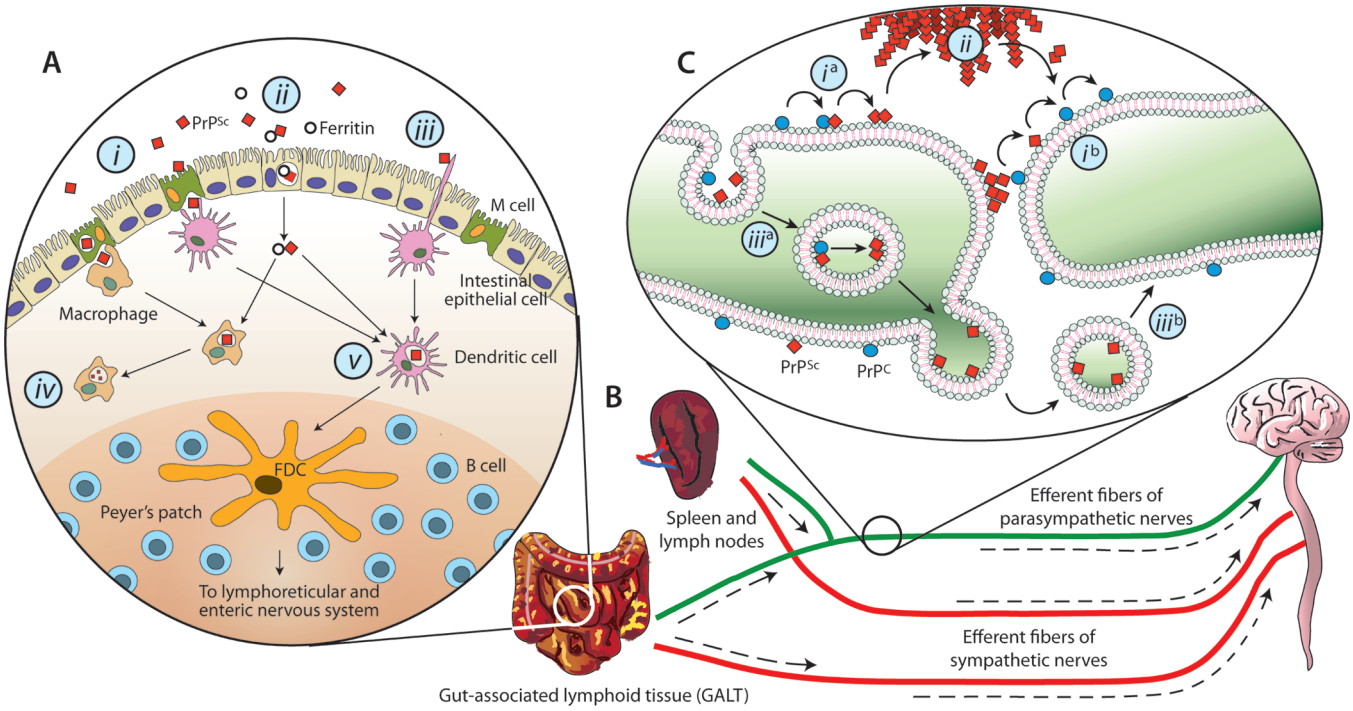

FIGURE 1: Schematic representation of the potential mechanism of neuroinvasion in transmissible spongiform encephalopathies. (A) Inital uptake of the TSE agent from the intestinal lumen has been proposed to occur through a number of alternative mechanisms, including M cell transcytosis (i), ferritin-dependent trancytosis through intestinal epithelial cells (ii), or via direct capture by dendritic cells (iii). While phagocytic cells such as macrophages appear to degrade PrP^{Sc} (iv), dendritic cells may deliver the TSE agent to follicular dendritic cells (FDCs) where early accumulation of PrP^{Sc} occurs (v). (B) After amplification of the TSE agent in lymphoid tissue such as the GALT and spleen, invasion of the nervous system is believed to proceed through peripheral nerves. Retrograde transport of the TSE agent is believed to occur along two distinct pathways, following efferent fibers of the sympathetic and parasympathetic nerves to the CNS. (C) Retrograde transport and propagation of PrP^{Sc} along neuronal processes may occur by stepwise interactions along the cell surface (i^a and i^b), via extracellular deposits (ii), or by vesicle-mediated mechanisms (iii^a and iii^b). See the text for details.

axolemmal surface, through a vesicle-mediated mechanism, or via free-floating extracellular aggregates (*36*) (Figure 1C). Once within the CNS proper, further retrograde transport of the TSE pathogen eventually results in infection of the brain, leading to characteristic spongiform degeneration and astroglial activation.

A variety of cellular factors have been implicated in the neuroinvasion of the TSE agent. With regard to initial uptake, association of ferritin with PrP^{Sc} has been reported to result in ferritin-dependent endocytosis and vesicular transcytosis by intestinal epithelial cell cultures (*37*) (Figure 1A). Members of the complement system (C1q, C2, C3, and factor B) may then play a role by enhancing initial transport of PrP^{Sc} to lymphoreticular cells (*7*). Polyanionic glycans such as heparin sulfate are another important player; they have been identified as a cell-surface receptor involved in prion uptake, as well as modulators of prion protein conversion and PrP^{Sc} propagation (*20*). Furthermore, cellular expression of another glycosaminoglycan-binding protein, laminin receptor (or its precursor) has been shown to be required for prion infectivity in cell culture, being implicated in both uptake and propagation of the TSE agent (see ref *20* and references cited therein).

As in other diseases of protein misfolding, a devastating neuronal pathology is associated with mammalian prion disorders. However, the precise nature of the toxic species and the mechanism of neurotoxicity remain unclear. The TSE disease states appear to be largely unrelated to a loss of PrP^{C} function, as PrP null mice suffer only mild cognitive impairment (*10*). While massive accumulation of protein aggregates would seem disruptive to normal cellular function, the extent of PrP^{Sc} deposition does not necessarily correlate with the severity of neurodegeneration (*38*). Furthermore, transgenic mice expressing some mutant forms of PrP spontaneously develop neurological disorders, but no infectivity is associated with prion protein aggregates accumulated in brain tissue of these animals (*39, 40*). Mice expressing GPI-anchorless prion protein, on the other hand, show high levels of infectious PrP aggregate deposits but a reduced level of neurodegeneration compared to prion-infected wild-type mice (*41*). Thus, it appears that infectious and neurotoxic forms of PrP could represent distinct proteinaceous species. This toxic PrP species may correspond to some intermediate or byproduct of the $PrP^{C} \rightarrow PrP^{Sc}$ conversion pathway. As appears to be the case with some other neurodegenerative disorders, small oligomers may represent such an intermediate, perhaps acting by compromising the integrity of cellular membranes through the adoption of porelike annular structures (*24*). While further studies are necessary to elucidate the precise identity and structural characteristics of the neurotoxic form of PrP, recent reports suggest a number of possible mechanisms leading to cell death in TSE diseases, including an increased level of NMDA receptor-mediated excitation (*42*), and activation of the Erk1/2 pathway (*43*).

MECHANISM OF PRION PROTEIN CONVERSION: LESSONS FROM STUDIES IN VITRO

The propagation of mammalian prions, intrinsically linked to conformational conversion of PrP^{C} to protease-resistant

PrP^{Sc}, was originally described by a heterodimeric refolding mechanism (*44*). This model proposes that PrP^{C} is thermodynamically less stable than PrP^{Sc}, but spontaneous conversion is kinetically limited. A critical step in the conversion would be formation of a heterodimer between PrP^{C} and PrP^{Sc} monomer, with the latter conformer acting as a monomeric template to induce a conformational conversion of PrP^{C}. However, to date there is little experimental evidence for a stable PrP^{Sc} monomer, and most available data indicate that prion protein conformational conversion and infectivity are ultimately associated with the aggregation process (*23, 24, 45*). A plausible model consistent with the latter view is the nucleated polymerization mechanism, according to which stabilization of the PrP^{Sc} conformation occurs only upon formation of an oligomer large enough to act as a stable nucleus (*46*). The monomeric protein would successively deposit onto this oligomeric nucleus, adopting the structure of PrP^{Sc}. The critical (rate-limiting) nucleation step is responsible for the "lag phase" observed in the spontaneous conversion reaction; this lag phase can be eliminated by "seeding" with a small amount of preformed PrP^{Sc} aggregates.

Early experimental evidence in support of such a polymerization-based conversion mechanism was provided by the finding that normal PrP^{C} can be converted into a PrP^{Sc}-like conformation by incubation with protease-resistant PrP (PrP-res) from infected animals (*47*−*49*). These cell-free studies established that aggregation is intrinsic to PrP conversion, showing that newly created protease-resistant PrP always remains tightly bound to the oligomeric PrP^{Sc} template (*47*−*49*). Furthermore, these experiments revealed two kinetically distinct steps in the prion conversion process: an initial binding event of PrP^{C} to the PrP-res template, followed by the conformational transition of bound PrP^{C} to the PK-resistant conformation.

Although highly specific, these early cell-free conversion reactions suffered from low yields, with the amount of newly generated protease-resistant material rarely exceeding the amount of input PrP^{Sc} (*47*−*49*). A major advance in the field was the development of a procedure called protein misfolding cyclic amplification (PMCA) which, using successive rounds of sonication and incubation, is able to indefinitely amplify the PrP^{Sc} conformer employing PrP^{C} present in brain homogenate as a substrate (*50*). Via serial dilution of PMCA reaction products into fresh brain homogenate, the original input PrP^{Sc} can be statistically eliminated, leaving only newly synthesized PrP-res aggregates. In a landmark study, the PMCA product amplified and serially diluted in this manner was demonstrated to cause TSE disease in animals (*15*). More recent work has shown that infectious material can also be generated by PMCA using purified PrP^{C} as a substrate (in the presence of small amounts of detergent, polyanions, and copurified lipids), lending strong support to the protein-only hypothesis (*16*).

While PMCA has emerged as an important tool in prion research, the mechanism by which this technique amplifies PrP^{Sc} remains unclear. Successive rounds of sonication are generally thought to increase the yield of amplification by fragmenting larger PrP^{Sc} aggregates into smaller species, increasing the number of loci available for the attachment of PrP^{C} substrate (similar to chaperone-mediated fragmentation involved in the replication of yeast prions in vivo as described above). However, it is entirely possible that sonication might also facilitate prion protein conversion by other means. For example, it could act as a source of energy required to overcome the activation barrier of prion protein refolding from the α-helical conformation of PrP^{C} to the β-sheet structure of PrP^{Sc}.

Insight from Studies with Recombinant Prion Protein. Since biophysical studies with brain-derived PrP pose major experimental challenges, many laboratories have resorted to bacterially expressed recombinant prion protein (rPrP) which can be readily purified in large quantities. Although the recombinant protein lacks both glycolsylation and the GPI anchor, its secondary and tertiary structures appear to be identical to those of brain-derived PrP^{C} (*51*). Thus, rPrP provides a useful tool for studying the physiochemical properties and conformational transitions of the prion protein. Early studies revealed that the transition of the recombinant PrP to an oligomeric β-sheet structure is especially effective at mildly acidic pH and in the presence of low concentrations of chemical denaturants (*52*). However, the α-helix → β-sheet conversion reaction described in these early studies was nonautocatalytic, failing to mimic self-propagation of infectious prions. Autocatalytic (seeded) conversion of rPrP was first generated by disulfide oxidation and reduction (*53*), leading to a model of prion propagation based on the domain-swapped structure as observed in a crystallographic study with human rPrP (*54*). There is, however, no evidence that disulfide reshuffling occurs during PrP^{C} → PrP^{Sc} conversion in vivo.

More recently, it was shown that rPrP can be converted to classical amyloid fibrils without the reduction of the native disulfide bond (*55*). While initial reports suggested that such conversion in vitro requires the presence of chemical denaturants or SDS (*55*−*59*), a recent study demonstrates that rPrP amyloid fibrils can also be formed in the absence of any denaturing agents or detergents (*60*). Akin to other amyloidogenic proteins (*25*), the conversion of rPrP to amyloid fibrils displays an initial lag phase, followed by a rapid phase of growth. The lag phase can be effectively bypassed by seeding with preformed aggregates, resulting in autocatalytic amyloid growth and indicating that formation of a stable nucleus is the rate-limiting step of the conversion process. However, the precise nature and molecular size of this nucleus remain unknown. Studies with other amyloid-forming proteins indicate that the conversion process is often best described by a double-nucleation mechanism which, in addition to primary nucleation (i.e., formation of elongation-competent nucleus), includes a second, fibril-dependent, nucleation step (*61*). In a study with the yeast prion protein, Sup35, it was shown that this secondary nucleation step corresponds to fibril fragmentation (*62*). Recent kinetic data indicate that a similar double-nucleation mechanism also applies to amyloid formation by human rPrP (A. C. Apetri and W. Surewicz, unpublished data).

Intermediates in Prion Protein Folding and Conversion. Among questions of fundamental importance to the understanding of prion protein conversion mechanism are those regarding the normal folding pathway of the prion protein and the identity of the direct monomeric precursor to aggregate PrP^{Sc}. The folding pathway of rPrP was extensively studied by kinetic methods. While early stopped-flow measurements suggested that the protein folds by a simple two-state mechanism (*63*), subsequent kinetic studies using both stopped-flow and continuous-flow methods indicate a

more complex folding pathway that can be described by a three-state model involving a monomeric intermediate (*64, 65*). Apart from kinetic data, the existence of a partially structured monomeric folding intermediate of the prion protein is also indicated by hydrogen−deuterium exchange and high-pressure spectroscopy experiments (*66, 67*), though the structural properties of this intermediate still remain to be characterized. Nevertheless, as compared to natively folded proteins, partially structured intermediates are typically characterized by an increased level of exposure of the polypeptide backbone to solvent and higher hydrophobicity, resulting in a high propensity for intermolecular interactions. Thus, the partially folded intermediate of the prion protein is a good candidate for a monomeric species that is directly recruited into the aggregated state associated with the conversion to β-sheet-rich PrP^{Sc} structure. Consistent with this view, it was found that the monomeric intermediate state for PrP variants associated with many familial prion diseases has increased stability and is thus more populated (*64, 68*).

Synthetic Prions? Intracerebral injection of recombinant PrP fibrils into transgenic mice overexpressing N-terminally truncated PrP^{C} was reported to result in a transmissible neurological disorder, though characterized by a very long incubation time as compared to those of classical TSE diseases (*14*). This observation has led to the conclusion that these aggregates represented "synthetic prions", associated with an unusually "slow" strain of the disease. While these findings are undoubtedly significant, there are a number of unresolved questions pertaining to this study. First, since the transgenic mice used greatly overproduce an N-terminally truncated form of PrP^{C}, it is possible that inoculation with rPrP fibrils could only accelerate a condition which would develop spontaneously with age. Second, the infectivity titer of recombinant amyloid appears to be very low, suggesting that only a tiny fraction of the converted material could be infectious. Indeed, relative to authentic PrP^{Sc}, recombinant PrP amyloid displays a significantly shorter PK-resistant core [with N-terminal cleavage sites at residues 152/153 and 162 (*57*) as compared to residue ~90 in PrP^{Sc}], clearly indicating a degree of structural nonequivalence between the molecular folds, at least in the N-terminal region.

The most obvious difference between recombinant and brain-derived prion protein is the presence of glycans and a GPI anchor in the latter. However, recent studies suggest that neither of these post-translational modifications is essential for the infectivity of the prion agent (*41, 69*). This indicates that specific backbone conformational features of PrP aggregates likely govern both their PK resistance and infectivity. In a recent PMCA study by Atarashi and co-workers, PrP^{Sc}-seeded conversion of the recombinant prion protein in the presence of a specific mixture of detergents was reported to result in aggregates with PK resistance similar to that of PrP^{Sc} (*70*). However the physiochemical properties and infectivity of these aggregates are yet to be characterized. While further investigation of these issues is required, generation of infectious prions from recombinant material and, thus, conclusive proof of the protein-only basis of TSE diseases appear to be tantalizingly close.

PRION STRUCTURAL BIOLOGY

Although it has been many years since fiber X-ray diffraction studies of amyloids formed by different proteins revealed a common cross-β structural motif (*25, 26*), progress in elucidating molecular details of these structures has been relatively slow. This is largely due to experimental difficulties associated with the structural study of large protein aggregates, as such polymers are generally not amenable to characterization by classical tools of structural biology such as crystallography and solution NMR spectroscopy. Nevertheless, in an important recent development, Eisenberg and co-workers have obtained X-ray diffraction data from microcrystals formed by a variety of short (four to seven residues) amyloidogenic peptides, including fragments of yeast and mammalian prion proteins (*71, 72*). These studies revealed atomic-level insight into the organization of β-strands within an amyloid-like conformation, showing that pairs of β-sheets associate with tight interdigitation of side chains at an anhydrous interface dubbed the "steric zipper" (Figure 2A, i). While similar crytallographic studies for larger polypeptides are not feasible, substantial progress in our understanding of the molecular organization of both yeast and mammalian prions has been made in recent years using techniques such as solid-state NMR spectroscopy, hydrogen−deuterium exchange, site-directed spin labeling, and electron microscopy

Yeast Prion Structures. The molecular architecture of amyloid fibrils formed by several yeast prion proteins has been recently studied using solid-state NMR spectroscopy and other methods. Amyloid fibrils formed by the prion domains of Ure2p, Rnq1, and Sup35 were found to share a common parallel and in-register β-structure motif (*73*) (Figure 2A, ii), although for Sup35 an alternative, β-helix-like model has also been proposed on the basis of fluorescence spectroscopy data (*74*). Parallel in-register β-structure, where single layers of individual molecules stack directly atop one another so that same residues are perfectly aligned, appears to be a common motif in many naturally occurring amyloids, having also been observed in fibrils formed by proteins such as Alzheimer's Aβ peptide (*26, 75*), α-synuclein, and tau (*76*). A notable exception is the structure for amyloid fibrils from the prion-forming domain of the HET-s protein from the filamentous fungus *Podospora anserina*. Here, a higher level of structural complexity is observed, where HET-s(218−289) forms a left-handed β-solenoid, with each molecule winding to form two three-stranded layers of the amyloid core (Figure 2A, iii) (*77*). Solid-state NMR data indicate that, compared to other fungal prions, fibrils of HET-s are characterized by an unusually high degree of order, a feature proposed to be related to its evolved biological function as a determinant of heterokaryon incompatibility in *P. anserina* (*77*).

Proposed Structures of Mammalian Prions. Low-resolution spectroscopic data indicate that the $PrP^{C} \rightarrow PrP^{Sc}$ conversion is associated with an increase in β-sheet content (*2*−*4*). However, neither the part of the protein involved in this conformational transition nor the specific structural features responsible for high proteinase K resistance of the entire C-terminal region starting at residue ~90 are clear. Nevertheless, several specific models for the structure of PrP^{Sc} and/or prion protein fibrillar aggregates have been proposed in recent years. The first of these models, guided by digital reconstitution of two-dimensional, p3 symmetry crystals sometimes observed in PrP^{Sc} isolates, postulates a β-helical structure (*78*). By threading a portion of the PrP sequence through a known β-helical fold, it was proposed that residues ~90−175 form left-handed β-helices

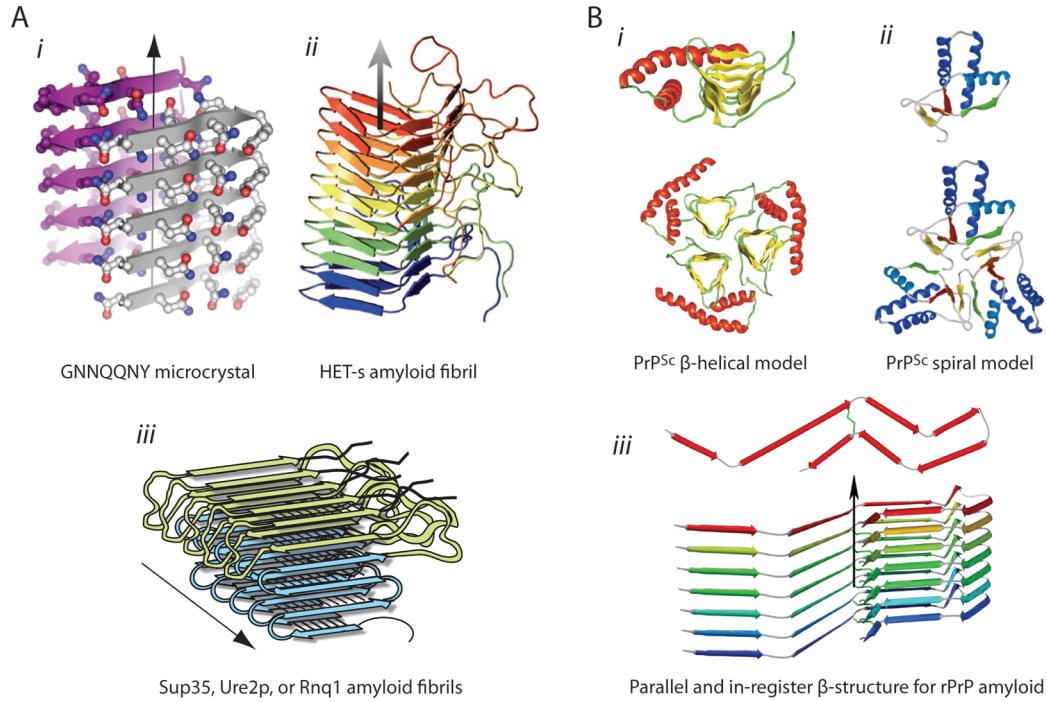

FIGURE 2: Comparison of structural models for fungal and mammalian prion protein aggregates. (A) Structural models of yeast fungal prion protein amyloids. (i) X-ray diffraction structure of microcrystals formed by the peptide GNNQQNY, corresponding to a fragment of yeast prion protein Sup35 (reproduced from ref *71*, reprinted with permission from Macmillan Publishers Ltd., Copyright 2005). The peptides form parallel and in-register β-sheets which associate at a dry interface known as the steric zipper. (ii) Left-handed β-solenoid structure determined by solid-state NMR for amyloid fibrils formed by yeast protein protein HET-s, where each molecule winds to form two three-stranded layers of the amyloid core (adapted from ref *77*, reprinted with permission from AAAS, Copyright 2008). (iii) General model for amyloid fibrils formed by yeast prion proteins Sup35, Ure2p, and Rnq1 (adapted from ref *73*, reprinted with permission from John Wiley and Sons, Inc., Copyright 2008). In all cases, solid-state NMR has revealed a parallel and in-register packing of individual molecules to form single layers where the same residues are perfectly aligned with their counterparts on neighboring molecules. (B) Structural models of PrP^{Sc} and/or PrP amyloid fibrils. (i) β-Helical model, where residues ~90−175 are shown to form left-handed β-helicies that associate into trimers, leaving the most C-terminal helices of monomeric PrP^{C} intact (reproduced from ref *78*, reprinted with permission from the National Academy of Sciences, Copyright 2004). (ii) Spiral model depicting the amyloid core as being comprised of a three stranded β-sheet and isolated β-strand, with complete retention of all three native α-helicies (adapted from ref *79*, reprinted with permission from the National Academy of Sciences, Copyright 2004). (iii) Parallel and in-register β-structure model determined experimentally for recombinant PrP amyloid fibrils (adapted from ref *59*, reprinted with permission from the National Academy of Sciences, Copyright 2007). In this model, residues ~160−220 form the PrP amyloid core (native disulfide bond colored green), with tight interdigitation of side chains. Individual monomers stack to form single-molecule layers so that the same residues are perfectly aligned. In all cases, arrows indicate the long fibrillar axis.

which associate into trimers, with the two C-terminal α-helices of the constituent monomers largely preserved (Figure 2B, i). A second model, based on molecular dynamics simulations, depicts a fundamentally different trimeric oligomer, which is postulated to stack in a spiral-like manner to form higher-order protofibrillar aggregates (*79*). Here, the amyloid β-core consists of a three-β-strand sheet, E1−E3 (residues 116−119, 129−132, and 160−164, respectively), and an isolated strand E4 (residues 135−140), with all three α-helices retaining their native monomeric conformation (Figure 2B, ii). Propagation of the protofibril in this model would occur by docking of PrP to the growing end through the E1−E4 interface.

In contrast to these two models, experimentally derived structural data for recombinant PrP amyloid suggest that prion protein conversion to amyloid fibrils involves major refolding of the entire α-helical domain. Indeed, two recent studies, using hydrogen−deuterium exchange (*59*) and site-directed spin labeling (*58*), consistently indicate that the β-sheet core of the human prion protein amyloid maps to the C-terminal part of PrP encompassing residues ~160/170−220. Distance information obtained from spin labeling studies demonstrated that residues within this core region form single-molecule layers that stack on top of one another with parallel and in-register alignment of β-strands, and a specific structural model consistent with these data has been proposed (Figure 2B, iii) (*58*).

Do any of these divergent structural models represent the infectious prion agent? While both the β-helical and spiral models are plausible, they are primarily theoretical, lacking direct experimental support. On the other hand, although the parallel and in-register β-structural motif is based on residue-specific distance constraints, its obvious limitation is that it has been derived on the basis of experiments with recombinant PrP amyloid fibrils. Compared with their brain-derived counterparts, these fibrils display reduced PK resistance for the segment of residues ~90−160 and show very little infectivity. Thus, the precise relation of available models to the structure of infectious PrP^{Sc} remains to be elucidated. However, the divergent models provided by structural studies may ultimately reflect the conformational variability of PrP aggregates associated with TSE disorders where, in addition to the existence of multiple prion strains, there are indications that neurotoxic and infectious PrP are distinct conformational species (see above). Clearly, despite recent advances, major

discoveries in structural biology of mammalian prions are still to be made.

SPECIES BARRIERS IN PRION TRANSMISSIBILITY AND PRION STRAIN DIVERSITY

Infection of one organism with prions generated in another species often results in delayed, if any, onset of disease, giving rise to the concept of so-called "species barriers" (Figure 3A,B) (*2, 4, 6*). For instance, hamster prions are considered to be essentially noninfectious to mice, while the transmission of mouse prions to hamsters, although more efficient, also results in an extended incubation time (*80*). After clinical disease has been established in the foreign host, however, subsequent passaging of prions isolated from this host through further individuals often results in "adaptation" to this new species, with a corresponding decrease in incubation times (*2, 4, 80*). What is the molecular basis of these effects?

Studies using transgenic animals have clearly demonstrated that TSE species barriers are closely related to the differences in prion protein sequence between donor and acceptor organisms (*81, 82*). Similar sequence specificity has also been shown in vitro using cell-free conversion assays (*49, 83*) and scrapie-infected neuroblastoma cells (*84*), revealing that these sequence-dependent effects are often governed by a few specific amino acids residues. The role of amino acid sequence in prion transmissibility has also been observed in yeast, best evidenced by the apparent barriers to [*PSI*+] transmission (resulting from fibrillization of the protein Sup35) existing between *Saccharomyces cerevisiae* and *Candida albicans* (*13*). Though the prion-forming domains of Sup35 from both species display the high glutamine/asparagine content associated with robust amyloid growth, it is typically difficult for Sup35 amyloid of one species to initiate conformational conversion in the other.

While these and many other observations clearly point to the importance of the prion protein amino acid sequence in TSE transmissibility between different species, recent observations indicate that the problem of transmissibility barriers is far more complex, confounded by the phenomenon of prion strains.

Prion Strains and Their Molecular Basis. Perhaps the most puzzling aspect of prion diseases is the existence of multiple strains of TSE agents that are associated with distinct disease phenotypes (distinguished by specific neuropathology, clinical symptoms, and incubation times) within the same animal species (*2, 4, 6*). Such phenotypic diversity within a single host species without variation in PrP genotype has for many years presented a major challenge to Prusiner's hypothesis that prions are just misfolded proteins. However, a growing number of observations now strongly indicate that multiple prion strains can be rationalized within the framework of this protein-only model, with individual strains representing distinct conformational states of the PrP^{Sc} aggregate. Crucially important initial evidence in this regard was provided by studies with so-called "hyper" and "drowsy" strains in hamsters: it was found that PrP^{Sc} associated with these two strains is characterized by a different PK-resistant core, clearly pointing to conformational differences (*85*). This conformational model was subsequently corroborated in

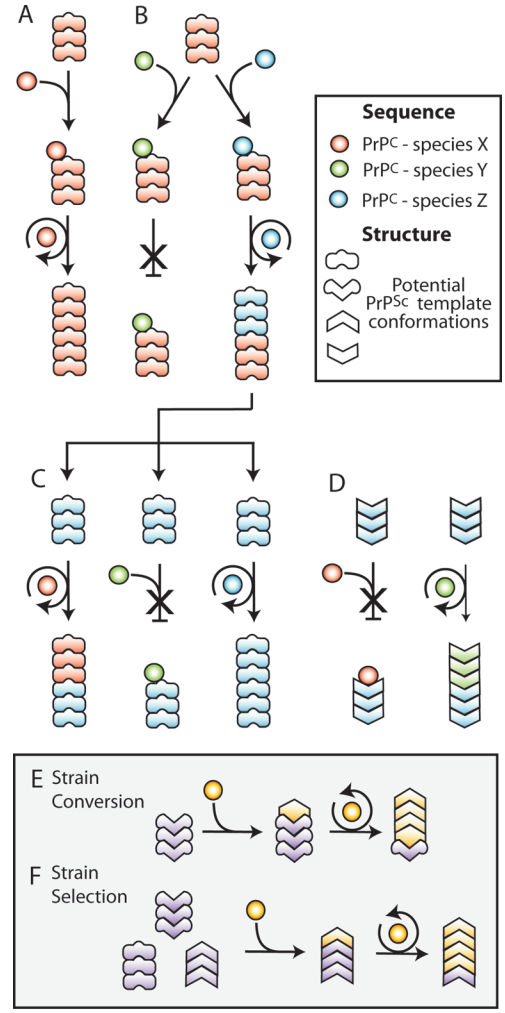

FIGURE 3: Illustration of the phenomenon of prion strains and transmissibility barriers. Different PrP^{C} sequences (differentiated by color) dictate the spectrum of allowable PrP^{Sc} conformations (depicted as different shapes), and these conformations represent different prion strains. For the sake of simplicity, transmission barriers are depicted as absolute, although in reality such barriers are often characterized by prolonged incubation times. (A) Infection of species X with a specific prion strain derived from the same species results in faithful propagation of strain characteristics. (B) Passaging of the same prion strain from species X to species Y and Z (which express nonhomologous PrP^{C}) may have different outcomes. If the PrP^{Sc} conformation of the donor strain from species X is not accessible to PrP^{C} of the host species, a barrier to transmission is observed as illustrated for species Y. On the other hand, if the conformation of the donor strain is accessible to the host PrP^{C}, transmission occurs, resulting in emergence of a new strain of prion in the host species (as illustrated by species Z). (C) The newly formed species Z prion strain often displays species-specific transmissibility characteristics similar to those of the original template. (D) Other species Z prion strains may, however, show transmission barriers that are distinct from those observed for the specific template-adapted strain shown in panel C. Thus, infectivity is associated with conformational properties of a particular prion strain. (E and F) Models of "strain switching", a phenomenon that may occur upon cross-species transmission of a specific prion strain. (E) Strain conversion model in which a PrP^{C} substrate adopts a conformation that is not identical to that of a nonhomologous PrP^{Sc} template. (F) Strain selection model in which a disease phenotype (strain) is associated with multiple PrP^{Sc} conformers, one of which is "dominant" in a particular species. Upon cross-species transmission, nonhomologous host PrP^{C} selects the PrP^{Sc} template most compatible with its amino acid sequence. In either case, new PrP^{Sc} conformations associated with the emerging strain may confer distinct transmission barriers and disease phenotype.

numerous studies showing strain-specific difference among PrP^{Sc} aggregates in terms of the exposure of certain epitopes (*86*), positions and intensities of infrared bands associated with β-sheet structure (*87*), and stability to denaturation by chaotropes such as guanidine HCl (*88*).

Strain diversity has also been observed in yeast prions, and studies with this system have provided irrefutable evidence for the purely conformational nature of this phenomenon (*11*−*13*). Importantly, for yeast prion protein Sup35, it was shown that different conformers of amyloid fibrils formed by this protein in vitro are sufficient to induce strain-specific phenotypes when introduced to living *S. cerevisiae* cells (*28, 29*). Since the infectious entity in yeast prions is represented by classical amyloid fibrils, this system is amenable to higher-resolution structural studies, allowing increasingly penetrating insight into the structural aspects of strain diversity (*89*). In fact, conformational polymorphism (i.e., ability to form different strains) appears to be a general feature of many amyloids; it has been observed, among others, for fibrils formed by Aβ peptide associated with Alzheimer's disease (*75*) as well as the recombinant mammalian prion protein and its fragments (*90, 91*).

Another factor contributing to the diversity of mammalian prions is glycosylation of asparagine residues at positions 181 and 197 (numbering according to human PrP sequence). Typically, different TSE strains are associated with distinct glycosylation patterns of PrP^{Sc} (i.e., distinct ratios of di-, mono-, and unglycosylated forms). Remarkably, these distinctive glycosylation patterns are maintained upon repeated passages in animals, indicating that different PrP^{Sc} aggregates can selectively recruit PrP^{C} monomers with similar glycoform ratios (*2, 4*). This could be explained within the framework of the conformational model described above by assuming that glycosylation patterns modulate the structure of PrP^{Sc} aggregates. However, it is at present unclear whether glycans affect the backbone conformation of protein molecules in PrP^{Sc} or alter packing of these molecules by introducing specific steric constraints. It has also been proposed that glycans modulate strain properties by forming crucial intermolecular contact sites between PrP monomers in PrP^{Sc} (*92*).

Role of Strains in Prion Transmissibility Barriers. While the phenomenon of prion strains is itself perplexing, an even more baffling aspect of prion biology stems from experimental observations that prion strains can also modulate interspecies transmissibility barriers (*4, 93*) (Figure 3C,D). For example, transmission studies of human prion diseases have shown that while classical Creutzfeldt-Jakob disease (CJD) prions may be efficiently transmitted to transgenic mice expressing human PrP^{C}, they encounter a significant barrier for transmission to wild-type mice. On the other hand, variant CJD prions (a new strain of human TSE disease believed to arise upon consumption of BSE-tainted beef) transmit readily to wild-type mice, whereas their transmission to transgenic mice expressing human PrP^{C} is relatively inefficient (see refs *4* and *93* and references cited therein). Thus, it appears that prions comprised of PrP with identical amino acid sequence but corresponding to different TSE strains may be characterized by pronounced differences with respect to transmissibility barriers. How could it happen?

This critically important question has been probed in many animal studies (*4*) as well in the experimentally more tractable system of yeast prions (*13*). Furthermore, some fundamental aspects of prion propagation, including strain diversity and transmissibility barriers, could be reproduced in vitro in a simple model of seeded fibrillization of a recombinant prion protein fragment (*90, 94*). The overall picture emerging from these studies is that transmission barriers and prion strains are closely related phenomena, resulting from the ability of prion proteins to misfold into multiple aggregate conformations. While a large spectrum of prion conformations is possible among different species, sequence variation guarantees that only subsets of these conformations are thermodynamically accessible in any given species. If the conformation of the specific donor strain is within the range of conformers accessible to prion protein of the host, transmission will occur. Importantly, this may happen regardless of whether the donor and acceptor of infection represent the same or different species. On the other hand, if the conformation of the incoming prion strain is outside the spectrum allowed to the host prion protein, a barrier to transmission will be observed. In other words, prion infectivity appears to be a conformational property of a particular prion strain. Amino acid sequence, on the other hand, dictates the spectrum of conformations accessible to prion aggregates from a particular host species; a transmission will occur only if this spectrum includes the conformation of a specific donor prion strain. Thus, prion strains and species barriers in prion transmissibility appear to be intricately related, representing two sides of the same coin.

While cross-species transmission often results in faithful propagation of the inoculating strain, in some cases it can result in strain switching, as observed in animal studies (*95*), yeast prion systems (*96*), and experiments in vitro (*97*). While the exact mechanism by which strain switching occurs is unclear, two models can account for this phenomenon in TSE diseases (*88, 93*). The first such model, dubbed strain conversion, proposes that host PrP^{C} can sometimes adopt a PrP^{Sc} conformation distinct from the nonhomologous template with which it is presented (Figure 3E). The second model stems from experimental data suggesting that multiple PrP^{Sc} structures are present within any individual strain (*98*), where the most abundant of these conformations presumably dictate the specific phenotypic disease state. If upon cross-species transmission this predominant conformation is inaccessible to host PrP^{C}, a less populous PrP^{Sc} structure may be amplified, resulting in a new distribution of PrP^{Sc} conformations (Figure 3F). This brief description of prion strains and transmissibility barriers can only scratch the surface of this complex problem. Interested readers should refer to an excellent recent review (*93*).

CONCLUDING REMARKS

In the nearly 30 years since Prusiner coined the term prion, an impressive collection of experimental evidence has emerged supporting the once heretical claim that misfolded proteins are solely responsible for the transmissible spongiform encephalopathies. Despite the considerable progress detailed above, however, our understanding of the unique prion pathogen is too often painted in broad strokes. Biochemical properties such as the cellular function of PrP^{C}, the origins of prion neurotoxicity, and the exact mechanism of PrP conformational conversion have proven to be notori-

ously elusive. Further detail in our knowledge of these topics will have implications not just for TSE disorders but also for more common neurodegenerative disesases such as Alzheimer's and Parkinson's diseases. An important key to unlocking many of the remaining mysteries lies in determining the precise structural underpinnings of the infectious PrP^{Sc} aggregates. While recent reports have offered intriguing clues, major discoveries in the structural biology of mammalian prions are still ahead of us. Recent successes in generation of infectious prions in vitro not only bring us tantalizingly close to final proof of the protein-only hypothesis but also may offer improved avenues for structural elucidation. A higher-resolution picture of PrP^{Sc} would prove to be invaluable in unraveling the precise molecular-level details surrounding the puzzling phenomenon of prion strains, and the conformational adaptability of PrP observed upon cross-species transmission.

ACKNOWLEDGMENT

We thank Jiri Safar for critically reading the manuscript.

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BI900108V