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The intriguing prion disorders

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Abstract. Prion diseases are among the most intriguing illnesses. Despite their rare incidence, they have captured enormous attention from the scientific community and general public. One of the most hotly debated issues in these diseases is the nature of the infectious material. In recent years increasing evidence has emerged supporting the protein-only hypothesis of prion transmission. In this model PrP^{Sc} (the pathological isoform of the prion protein, PrP^{C}) represents the sole component of the infectious particle. However, uncertainties about possible additional factors involved in the conversion of PrP^{C} into PrP^{Sc} remain despite extensive attempts to isolate and characterize these elusive components. In this article, we review recent developments concerning the protein-only hypothesis as well as the possible involvement of cellular factors in PrP^{C} to PrP^{Sc} conformational change and their influence on the pathogenesis of prion diseases.

Keywords. Creutzfeldt-Jakob disease, transmissible spongiform encephalopathies, prion, cellular conversion factors.

Introduction

Transmissible spongiform encephalopathies (TSEs), also known as prion disorders, include several neurological diseases, such as Creutzfeldt-Jakob disease (CJD), fatal familial insomnia (FFI), Gertsmann-Straussler-Scheinker syndrome (GSS) and kuru in humans [1, 2]. In other mammals, bovine spongiform encephalopathy (BSE) is found in cattle, scrapie in sheep and goats, and chronic wasting disease (CWD) in elk and deer [1, 2]. Although the clinical symptoms vary in distinct diseases, they usually include dementia and/or ataxia with progressive loss of brain function, irreversibly resulting in death [3]. The hallmark of prion diseases is the misfolding of the prion protein observed in the brain of affected individuals [1]. Misfolded proteins have the intrinsic tendency to form large extracellular aggregates and fibrillar structures that may in turn form amyloid plaques in a fashion similar to that observed in Alzheimer's and Parkinson's diseases, and many other protein misfolding disorders [4].

In humans, TSEs are divided into sporadic, familial and infectious forms. Sporadic Creutzfeldt-Jakob disease (sCJD) is the most common human TSE, accounting for 90 to 95% of cases and affecting mostly individuals over 60 years of age. sCJD has an incidence of one to two new cases per million people each year [5]. Hitherto, no epidemiologic factors or genetic linkages have been found to be associated with this form of the disease. All familial cases have been shown to arise from mutations identified in the sequence of the gene encoding for the prion protein (Prnp) [2]. More than 20 different mutations have been reported that mostly involve amino acid substitutions and, to a lesser extent, insertion of an additional copy of an eight-amino acid sequence repeat in the N-terminal extremity of the prion protein [6]. Severity and age of onset are variable depending on the position and type of

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mutation. It has been hypothesized that mutations may destabilize the native structure of normal prion protein (PrP^{C}), leading to its misfolding into a protease-resistant form (PrP^{Sc}). But proof of this concept has yet to be demonstrated. Familial CJD, and GSS syndrome, accounts for 5–10% of human prion disease cases and exhibits autosomal dominant transmission [6]. Infectious forms of the disease represent less than 1% of reported cases and include kuru, iatrogenic CJD and the recently described variant CJD (vCJD). Kuru was spread by ritual mourning cannibalism among New Guinea tribesmen, the outbreak reaching its peak in the mid 1950s and gradually decreasing upon cessation of cannibalism [7]. However, cases were still being reported in the 1990s, demonstrating that prion disease incubation time can exceed 40 years [8]. Iatrogenic cases of prion diseases have been reported and consist of transmission of the disease through the use of contaminated human-derived products or incomplete decontamination of surgical instruments [9]. The majority of iCJD cases have come from hormone therapy gathered from cadaveric human pituitary glands and dura mater collected from affected individuals [10]. Finally, vCJD emerged in the mid-1990s following human consumption of cattle affected by bovine spongiform encephalopathy (BSE) [11]. Although this disorder is very rare, it has drawn considerable attention from the public and led to severe economic and political consequences in Europe and in the United States. The two main reasons for this impact include the unique nature of the infectious agent and the fact that it is impossible to accurately estimate the number of upcoming cases of vCJD due to the very long incubation time of the disease in humans [12–14].

In this review we will discuss the structure and biochemical characteristics of both PrP^{C} and PrP^{Sc} and provide an update of the latest data concerning the protein-only hypothesis. We will also review in detail the potential mechanisms and cellular factors involved in prion conversion. Here, we refer to PrP^{Sc} as the misfolded form of PrP, which has been shown to be infectious; PrP^{res} is used to refer to the proteinase K-resistant form, which has not been proven to be infectious.

Structure and properties of PrP^{C} and PrP^{Sc}

The nature of the infectious agent responsible for TSE has been the subject of intense debate over the past decades [15]. Initially, the infectious agent was thought to be a virus with an extraordinarily long incubation time. But the fact that it resisted conventional anti-viral inactivation procedures [16] led to the hypothesis that the infectious agent is devoid of nucleic acid and instead consists of a replicating protein [17]. In 1982, Prusiner and co-workers isolated a protease-resistant glycoprotein and proposed that it was the active component of the infectious agent, which they called prion (for proteinaceous infectious particle) [18]. The characterization of the gene encoding for the prion protein along with structural and biochemical studies during the mid-1980s started to reveal the unorthodox and fascinating aspects of prion biology [19–21]. One of the most surprising particularities of the prion protein is its ability to be folded in at least two isoforms, PrP^{C} being the normal protein and PrP^{Sc} being the pathologic conformation (where C stands for cellular and Sc for scrapie). The two isoforms consist of the same amino acid sequence and have not been shown to contain any different chemical post-translational modifications [22]. The structural change from PrP^{C} to PrP^{Sc} consists of a drastic alteration of the structure as well as the biochemical properties of the protein [23]. Indeed, PrP^{C} secondary structures contain 42% alpha helix and 3% beta sheet. Upon conversion into PrP^{Sc}, the beta-sheet structure becomes prominent, with 43 vs. 30% for alpha helix [24, 25]. As a result of the structural differences, PrP^{Sc} is insoluble and relatively resistant to proteases, while PrP^{C} is soluble and protease sensitive.

The prion protein is well conserved among species and displays overall similar structures observed through nuclear magnetic resonance (NMR) spectroscopy of recombinant proteins [26–29]. The polypeptide comprises 253 amino acids before post-translational modifications. Maturation of the protein involves cleavage of the N-terminal end by a signal peptidase in the endoplasmic reticulum (ER), the removal and replacement of the amino acid sequence 232–253 by the glycosylphosphatidylinositol anchor, formation of a disulfide bridge between two cysteine residues and glycosylation of two asparagines (Fig. 1) [1, 30]. The mature human PrP^{C} consists of 209 amino acids, the length varying slightly in different species, mostly due to the number of repeats of the aforementioned eight-amino acid sequence (the octapeptide repeat region) localized in the N-terminal region [6]. Mature PrP^{C} can be divided in two distinct regions: one flexible N-terminal region that is essentially unstructured and comprises amino acids 23–125; and a C-terminal region comprising amino acids 126–231, composed of three alpha-helical structures and a short beta-sheet motif. Helices 2 and 3 are stabilized by a disulfide bond between cysteine 179 and cysteine 214 [31]. PrP^{C} is a glycoprotein that may contains two N-linked oligosaccharide chains at asparagine residues 181 and 197 for human and N180 and N197 for mouse PrP^{C}. In Syrian hamster, more than 50 different sugar chains have been shown to be attached to PrP^{C} [32–34]. Oligosaccharide chains are added in the ER, further modified and extended to contain sialic acid in the Golgi (Fig. 1). Although PrP^{C} possesses two glycosylation sites, the protein is found as a mixture of mono-, di- or unglycosylated forms, depending on the neuronal region and species [35]. But the physiological significance of these differences remains unknown. The fact that PrP^{C} glycosylation

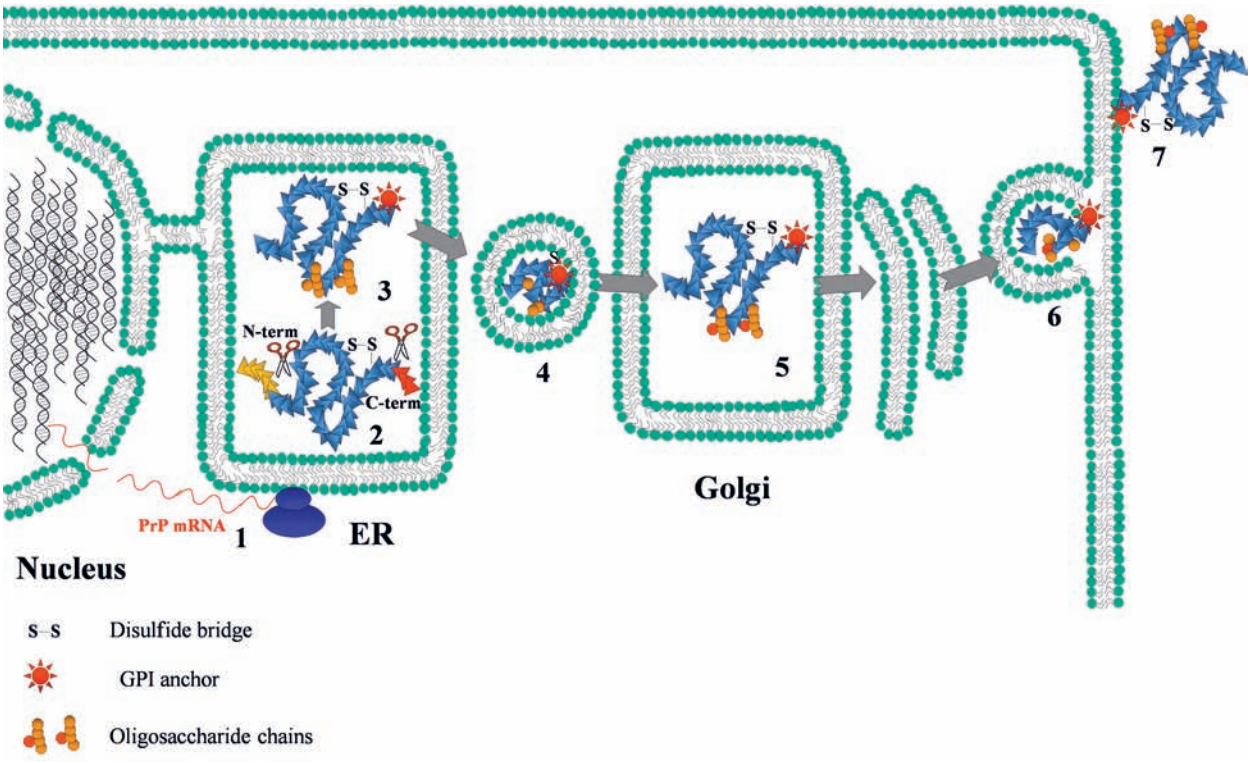

Figure 1. Biosynthesis of mature PrP^C. mRNA is translocated from the nucleus and translated by ER-associated ribosomes into the precursor protein (1). The 23-amino acid N-terminal signal peptide directs the polypeptide to the ER, where PrP^C undergoes several post-translational modifications: removal of the signal sequence, removal and replacement of the C-terminal end by the GPI anchor, formation of a single disulfide bridge and optional N-glycosylation of two asparagine residues (2–3). PrP^C is transported from the ER to the Golgi, where N-linked oligosaccharides are modified to produce the mature and complex sugar types (4–5). Mature PrP^C is then trafficked to the lipid raft domain of the membrane, where it is attached to the outer leaflet through the GPI anchor (6–7).

is conserved in mammals argues for an important role of the sugar moieties.

Another characteristic of PrP^C is the presence of a glycosylphosphatidylinositol (GPI) anchor at the C-terminal end of the polypeptide. This tail is added in the ER, following cleavage of the hydrophobic C-terminus fragment, and it enables PrP^C to be targeted and attached to the exterior leaflet of the cell membrane (Fig. 1) [30, 36]. PrP^C is found mostly in the cholesterol- and sphingolipid-rich membrane domain, also known as the lipid raft [37–39]. However, part of the PrP^C pool is constitutively present outside of the lipid raft domain, and internalized via clathrin-mediated endocytosis. Some of the protein molecules are recycled to the cytoplasmic membrane [30, 40]. This type of endocytosis is unusual for a GPI-anchored protein since PrP^C is devoid of a cytoplasmic domain that usually recruits clathrin-coated pits. This may suggest that some unknown proteins can interact with PrP^C and function as an adaptor to enable PrP^C to be internalized via clathrin-mediated endocytosis [41]. That may be important since the exact conversion site is currently unknown. Electron microscopy studies of both scrapie-infected N2a cells and brain tissue have shown that PrP^{Sc} is observed in late endosomes and lysosomes [42, 43]. Thus, it cannot be ruled out that, conversion may be initiated in these organelles by virtue of acidic pH and then further amplified in the lipid raft. Interestingly, low pH has been shown to favor aggregation of recombinant PrP^C into PrP^{Sc}-like structures [44–46].

The function of PrP^C remains largely unknown. Over the past decade several possible physiological functions of the protein have been proposed [47, 48]. Not surprisingly, as it is found in signaling molecules-rich lipid rafts, PrP^C has been shown to be involved in a signal transduction pathway leading to neuroprotection [49–51]. Another widely studied putative function of PrP^C concerns the binding and metabolism of copper [52]. A role in normal brain copper metabolism is suggested by the finding that the octapeptide repeats of PrP^C are able to bind copper within the physiological concentration range [53, 54]. In animal models, significant changes have been detected in the levels of brain copper in scrapie-infected mice, before the onset of clinical symptoms [55, 56]. Furthermore, in human sporadic CJD there is a decrease of up to 50% in brain copper levels [56]. PrP knockout mice display lower copper levels, but unaltered concentrations of iron and zinc in the synaptosomes [54]. However, constitutive and conditional PrP knockout mice are viable and show

no major physiological or behavioral changes compared with wild-type animals, suggesting that PrP^{C} may not be an essential protein, at least in mice [57–59].

The prion hypothesis

The protein-only hypothesis postulates that PrP^{Sc} is the infectious particle responsible for prion propagation and that it can replicate by inducing the autocatalytic conversion of PrP^{C} into its scrapie isoform [1]. This hypothesis gained great support with the finding that highly purified PrP^{Sc} produces the disease when injected into wild-type animals [18] and with the discovery that PrP knockout mice are resistant to prion infection [57]. Neverless, skeptics argue that definitive proof, consisting of *in vitro* generation of infectivity by misfolding of the prion protein, is largely missing [15, 60].

Several strategies have been followed in order to definitively probe the prion hypothesis [15]. One of the most studied, but thus far unsuccessful, has been to generate infectious mammalian prions starting from PrP^{C} harboring several mutations found in familial TSE-affected patients. Even though some properties similar to PrP^{Sc} were found among several mutant PrPs tested [61, 62], none of them have been shown to be infectious in animals. Another strategy has been based on the *in vitro* induction of full-length or truncated recombinant PrP protein misfolding, as well as synthetic fragments of the polypeptide [15]. Although several synthetic polypeptides were shown to harbor PrP^{Sc}-like properties (e.g. formation of aggregates, enriched beta-sheet structures etc.) [44, 63–66], none of the constructs tested have been able to induce TSE-like disease in wild-type animals. Recently, a recombinant PrP fragment lacking the N-terminal one-third of the polypeptide was assembled *in vitro* into amyloid fibrils and was found to induce a TSE-like disease when injected in transgenic mice overexpressing the same truncated portion of PrP [45]. While this finding brings additional support for the prion hypothesis, it poses several problems, a major one being the fact that the disease was observed in animals overexpressing a truncated PrP protein and not wild-type mice. This is significant since it has been shown that transgenic animals overexpressing PrP may spontaneously develop a prion-like disease [62, 67, 68]. One of the latest and most solid bits of evidence in favor of the prion hypothesis consists in the demonstration that PrP^{Sc} generated *in vitro* by cyclic amplification of the misfolding event was shown to be infectious in wild-type Syrian hamsters [69]. However, this experiment still could not completely rule out the involvement of other components in the infectious units since PrP^{Sc} was formed in crude brain homogenate.

Factors involved in prion conversion

Even though the body of evidence in favor of the prion hypothesis is very compelling, alternative models have been suggested, involving viruses, virinos and other infectious agents containing small RNAs [60, 70–72]. In particular the putative participation of nucleic acids as part of the infectious particle is still under consideration. Retroviral RNA has been shown to co-sediment with PrP^{Sc} [73, 74], and short (<4 kb) RNA fragments are released after nuclease digestion from purified infectious fractions [75]. Several reports have demonstrated that PrP^{Sc} can interact with RNA with variable affinity [76–78]. However, the specificity of these interactions still remains to be established, as recently described [79].

One of the most important issues concerning possible component(s) other than PrP involved in prion transmission is to distinguish between factors that are part of the infectious particle, as opposed to cellular factors that are involved in the conformational change. If the additional factors must be part of the infectious particle, then the infectious units would not be composed solely of PrP^{Sc}. Alternatively, additional factors may need to be present in the host to sustain proper prion replication. These factors may be cellular components presumably engaged in other functions in the infected cells that accidentally participate in prion conversion. In the latter case, these additional factors (termed conversion factors) should not be considered part of the infectious particle, but rather host-encoded molecules that aid prion replication.

Some evidence supports the existence of conversion factor(s) in the prion replication process. The existence of host factor(s) was first suspected when transgenic mice expressing both human and mouse PrP^{C} were challenged with human prions. Surprisingly, mice co-expressing both proteins were resistant to prion replication, while mice expressing only human PrP^{C} ($HuPrP^{C}$) developed the disease following human PrP^{Sc} inoculation [80]. This suggested that mouse PrP^{C} ($MoPrP^{C}$) was able to inhibit the conversion when co-expressed with $HuPrP^{C}$. Interestingly, transgenic animals expressing a chimeric protein consisting of pieces of the human and the mouse gene were also susceptible to infection with human prions [81]. This result enabled the authors to conclude that $MoPrP^{C}$ inhibited the conversion of $HuPrP^{C}$ by binding to an additional factor. Further studies performed by the same group showed that the host factor, termed protein X, was able to bind PrP^{C} through its C-terminal end [82].

Other evidence supporting the involvement of conversion factor(s) includes genetic studies in mice suggesting that other loci besides Prnp (the gene encoding for PrP) may modulate the time course of disease in PrP^{Sc}-innoculated animals [83]. In addition, biochemical studies of cell-free conversion of PrP have shown that partially purified hamster PrP^{C} is not converted when mixed with purified

PrP^{Sc}; on the other hand, conversion is restored when the cell lysate is added to the sample [84, 85]. Similar results have been obtained using our recently described PMCA (protein misfolding cyclic amplification) technology [K. Abid and C. Soto, unpublished results] and suggest that unknown factors present in brain homogenate are essential for the conversion. Finally, data in the chronically infected mouse neuroblastoma cell line N2a indicate that some cell clones can sustain prion replication, while others cannot [86, 87]. Interestingly, the expression levels or subcellular localization of PrP in cells sensitive and resistant to infection appear to be the same [K. Maundrell and C. Soto, unpublished results]. These data might be interpreted as suggesting that some cell clones express the appropriate quantities of the conversion factor, whereas others do not.

While the nature of the conversion factor remains elusive and it is also unknown whether the factor is one single molecule or several different ones, several compounds have been shown *in vitro* to bind PrP and promote prion replication. Sulfated glycans have been shown to be able to bind PrP^{C} [88], to stimulate PrP^{res} formation [89] and have been observed in PrP amyloid plaques from scrapie-infected mice [90]. Conversely, heparinase III treatment of infected cells in culture diminish levels of PrP^{res} [91]. Small, highly structured RNA, vertebrate RNA, and homopolymeric nucleic acids such as poly(A) and poly(dT) or nonspecific DNA have been shown to facilitate prion conversion *in vitro* from recombinant or hamster brain PrP^{C} [85, 92–94].

Since the lipid raft harbors both isoforms of the protein [38], it has been suggested that these domains rich in cholesterol and sphingolipids may harbor the mysterious factor(s). Even though no proteinaceous determinant present in these domains has been proven to be absolutely essential for conversion, the cholesterol level in the lipid raft appears to modulate the process [39] [K. Maundrell and C. Soto, unpublished results]. Interestingly, replacing the GPI tail with a transmembrane CD4 protein domain or by a segment of the Qa protein (two proteins located in the membrane but not in the lipid raft domains) prevents the lipid raft–PrP association, and results in significant reduction or abrogation of the conversion [39, 95]. However, a recent report showed that in scrapie-infected transgenic mice expressing PrP lacking the GPI membrane anchor, abnormal protease-resistant PrP^{Sc} was deposited as amyloid plaques, rather than the usual nonamyloid form of PrP^{Sc} [96]. Although PrP^{Sc} amyloid plaques induced brain damage reminiscent of Alzheimer's disease, clinical manifestations were minimal.

Finally, it is likely that small ions may also modulate prion conversion. As mentioned in the previous section, PrP^{C} is able to bind copper through its octapeptide sequence repeat, but the effect of copper at the molecular level and on conversion efficiency is unclear. Copper has been shown to recover infectivity of partially denatured PrP^{Sc} [97] and to enhance PK resistance of PrP^{res} [98]. Moreover, copper chelation has been reported to delay the onset of prion disease in animals [99]. Conversely, copper has been shown to prevent infection of N2a cells [100] and inhibit *in vitro* aggregation of recombinant PrP^{C} into amyloid fibrils [101]. More recently, Supattapone's group have reported that *in vitro* PrP^{res} amplification was inhibited by $CuCl_2$ and $ZnCl_2$ at IC50 (mean inhibiting) concentrations of approximately 400 nm and 10 μm, respectively [102]. We have observed similar results using our PMCA technology [P. Saa, J. Castilla and C. Soto, unpublished results].

Models of conversion

The molecular basis of the PrP conversion mechanism is not completely understood. Several models have been proposed and revised on subsequent findings. The 'seeding/nucleation model' proposes that monomeric PrP^{Sc} exists in equilibrium with PrP^{C} (Fig. 2) [103, 104]. In this scenario, monomeric PrP^{Sc} would represent a minor and transient isoform of PrP and would be stabilized only when forming ordered aggregates. The stabilized oligomers act as nuclei to recruit monomeric PrP^{Sc} into the polymer in a process that is much faster than the initial formation of the seed. The 'template-assisted model' proposes that PrP^{Sc} contains the refolding instruction that is applied to PrP^{C} upon interaction of the two isoforms catalyzed by the protein X and mediated by the formation of a conformational intermediate [23]. The conversion process first implies the formation of a heterodimeric PrP^{C}-PrP^{Sc} unit that would initiate the conformational change of PrP^{C}, becoming homodimeric PrP^{Sc}, as depicted in Figure 2. The latter would subsequently interact with other PrP^{Sc} dimers and eventually form larger aggregates. In the template-assisted model, the infectious unit is a monomer of PrP^{Sc}, and the formation of larger aggregates is not needed for prion replication. Recently this model was challenged by Caughey and colleagues who showed that small oligomers composed of less than six units of PrP^{Sc} were noninfectious in Syrian hamsters [105]. In fact, the particles harboring the highest infectious potential were non-fibrillar structures composed of 14–28 units of PrP^{Sc}. Similarly, in recent years, evidence suggests that large aggregates found in various neurodegenerative diseases such as Alzheimer's disease and other amyloidoses might also be relatively inert and less responsible for the expansion of the disease compared with smaller aggregates [4, 106]. Fibril formation might be a protective strategy to sequester the harmful oligomers rather than the culprit of neurodegeneration [106]. Thus, growing evidence supports the hypothesis that small aggregates of PrP^{Sc} rather than monomers or large fibrillar structures can catalyze the conversion of PrP^{C}.

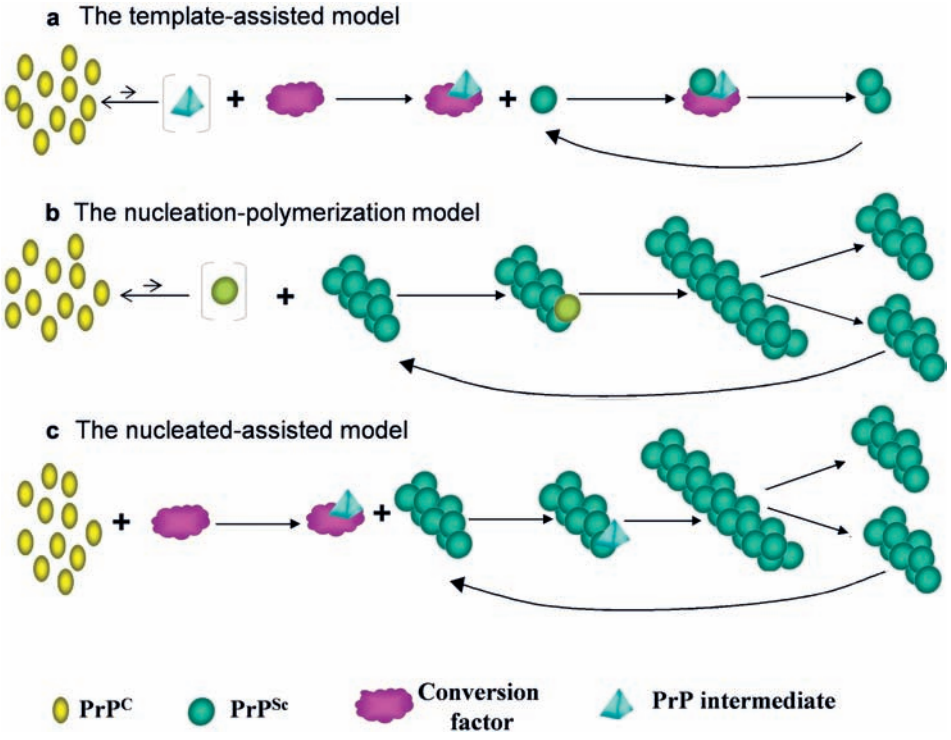

Figure 2. Different models for prion conversion. (*a*) The template-assisted hypothesis proposes the interaction of a PrP^{C} intermediate with a monomeric form of PrP^{Sc} and the subsequent conversion of PrP^{C} into PrP^{Sc}. The PrP^{Sc} dimer would then interact with other dimers and thus form larger aggregates. (*b*) The seeding-nucleation hypothesis proposes that PrP^{C} and PrP^{Sc} are in dynamic equilibrium, but monomeric PrP^{Sc} is unstable and become stabilized by the formation of an oligomer that acts as a seed to bind and further stabilize PrP^{Sc} monomers, displacing the equilibrium to the accumulation of the pathological isoform. (*c*) An alternative model, the nucleated-assisted hypothesis, proposes that PrP^{Sc} never exists as a monomer, but requires two subsequent structural rearrangements to form the oligomeric PrP^{Sc} species. The first step in this model is the formation of a partially unfolded intermediate (PrP*) upon interaction with an endogenous conversion factor. This structural change results in the exposition of hydrophobic fragments to the solvent, facilitating the interaction with other PrP*. This interaction results in further structural changes of the protein to adopt an intermolecular beta sheet. If enough PrP* is present, it is possible to form a stable oligomer containing a minimum number of molecules of the protein. This stable oligomer corresponds to PrP^{Sc}, which can then act as an infectious molecule, recruiting PrP* and catalyzing prion conversion.

Based on recent biochemical experiments of prion conversion, we would like to propose a new model of prion replication, termed the nucleated-assisted model (Fig. 2). A key event in this model would be the formation of an intermediate structural state (PrP*) upon binding of PrP^{C} to the conversion factor. The intermediate conformation would enable and prepare PrP^{C} to sustain the profound structural changes leading to PrP^{Sc}. The existence of intermediate states has been the subject of numerous studies [107, 108]. Intermediate states have been described when solution conditions are altered, such as in the presence of metal ions or upon changes in pH [109]. However, the physiological relevance of these intermediates remains to be established. It has long been postulated that mutations along PrP^{C} can destabilize the overall structure of the polypeptide and therefore facilitate the conversion. It is tempting to speculate that a mutant protein could be more unfolded than wild-type PrP^{C} and therefore be more prone to adopt multiple conformations, one of which could represent an intermediate 'convertible isoform'. The next key step in the nucleated-assisted model would be the further structural rearrangement of the protein and its stabilization upon intermolecular interactions with other molecules of PrP*. The formation of the minimum stable oligomer would be the limiting step for the 'de novo' formation of PrP^{Sc}. However, in infectious forms of TSE, the infectious agent corresponding to an oligomer of PrP^{Sc} would catalyze the further conformational changes of PrP* by incorporating the protein into the growing aggregate. In this model there is no equilibrium between PrP^{C} and PrP^{Sc}, and the latter isoform exists only as an oligomer or larger polymer.

Concluding remarks

In recent years, increasing evidence has emerged to support the prion hypothesis. The discipline has never been so close to confirming that PrP^{Sc} is indeed the only component of the infectious agent. One of the main reasons for this progress lies in the fact that *in vitro* systems to study prion conversion have been improved and opti-

mized since the pioneering work of Caughey and coworkers, who were the first to study prion replication in test tube assays [104]. One of the most interesting issues in prion biology is the possible involvement of additional factors during the conversion of PrP^{C} into PrP^{Sc}. Our current conversion models harbors two main uncertainties: the existence of the conversion factor and the nature of intermediate forms required for conversion. The discovery of either one may boost the discovery of the second, since the conversion factor may be responsible for shaping the intermediate structure. *In vitro* models of PrP conversion are an invaluable tool in helping to uncover the molecular details of prion replication by allowing reconstitution of PrP^{Sc} propagation using purified components. These discoveries would not only allow a better understanding of the conversion process itself, but would open new avenues for novel therapeutic strategies against prion diseases.

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