Prion protein conversions: insight into mechanisms, TSE transmission barriers and strains

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Conversion of PrP\(^C\) to aberrant forms such as PrP\(^\text{Sc}\) appears to be critical in the transmission and pathogenesis of transmissible spongiform encephalopathies (TSEs) or prion diseases. In vitro studies have shown that TSE-associated, protease-resistant forms of PrP can cause PrP\(^C\) to convert to forms that are similarly protease-resistant under a wide variety of conditions. These observations have provided evidence that pathological forms of PrP have at least limited capacity to propagate themselves, which is necessary for them to be infectious. PrP conversion reactions have proven to be highly specific and appear to account, at least in part, for TSE species barriers and the propagation of strains. Such in vitro conversion systems have yielded insights into the molecular mechanisms of TSE disease and are being exploited as screens for anti-TSE drugs and as bases for diagnostic tests.

The conversion of PrP\(^C\) to neuropathological forms is central to the pathogenesis of transmissible spongiform encephalopathies (TSEs) or prion diseases. Hence, strategies for coping with these diseases usually depend on understanding PrP conversion and how it relates to fundamental TSE disease parameters such as the spread of infection, species barriers, the propagation of strains and neurotoxicity. As detailed elsewhere in this volume, the primary difference between PrP\(^C\) and the scrapie-associated form PrP\(^\text{Sc}\), appears to be conformational. At the cellular level in the case of scrapie-infected cells, PrP\(^\text{Sc}\) is formed post-translationally after mature PrP\(^C\) is translocated to the cell surface (see other chapters in this volume)\(^1,2\). A critical question is what factor(s) control the conversion of PrP\(^C\) to PrP\(^\text{Sc}\) and the propagation of TSE infectivity? According to protein-only or prion hypotheses, PrP\(^\text{Sc}\) is the infectious agent that propagates itself by causing the conversion of PrP\(^C\) to PrP\(^\text{Sc}\). Although the mechanism and full neuropathological implications of PrP\(^\text{Sc}\) formation in vivo remain uncertain, studies of PrP conversion under a variety of conditions have provided important insights into the ways in which PrP\(^C\) can adopt a PrP\(^\text{Sc}\)-like protease-
resistant state upon interaction with PrP<sup>Sc</sup>. Related studies have revealed ways in which PrP<sup>C</sup> can misfold spontaneously as a result of treatment with denaturants and/or introduction of TSE-associated mutations (reviewed by Alonso et al<sup>3</sup> and Glockshuber<sup>4</sup>). The spontaneous misfolding of PrP<sup>C</sup> is likely relevant to the instigation of sporadic and familial TSE diseases. On the other hand, PrP<sup>Sc</sup>-induced misfolding of PrP<sup>C</sup> appears to be fundamental to TSEs of infectious origin and to the propagation of spontaneously formed PrP<sup>Sc</sup> in sporadic and familial TSE diseases. This chapter will focus on the understanding of PrP<sup>Sc</sup>-induced misfolding of PrP<sup>C</sup> and its relevance to TSE diseases and prospective therapies.

First, a few words about nomenclature: Although pathological forms of PrP are often designated PrP<sup>Sc</sup>, for PrP-scrapie, there is considerable variation in the properties of abnormal TSE-linked forms of PrP in terms of protease-resistance, association with infectivity and, apparent neurotoxicity. Here, the term PrP<sup>Sc</sup> is used to refer to scrapie-associated PrP and PrP-res to designate PrP isoforms that exhibit the usual partial protease resistance of PrP<sup>Sc</sup>, whether they are scrapie-associated or not. The term PrP<sup>C</sup> is used to refer to PrP in its normal structure and conformation and the term PrP-sen to refer generically to protease-sensitive forms of PrP, whether normal (<i>i.e.</i> PrP<sup>C</sup>) or not (<i>e.g.</i> various recombinant forms).

**Basic binding and conversion interactions**

PrP<sup>C</sup> is most commonly found as glycosylphosphatidylinositol (GPI)-linked membrane glycoprotein that is soluble as a monomer in mild detergents. When incubated with PrP<sup>Sc</sup> or other types of PrP-res, the PrP-sen tends to bind to the PrP-res in a protease-sensitive state and then more slowly convert to a protease-resistant state like that of the original PrP-res<sup>5–7</sup>. This reaction has been shown to occur under a variety of conditions. The simplest, most biochemically defined reactions contain mixtures of largely purified PrP-sen and PrP-res preparations and can be stimulated by chaotropes, detergents and/or chaperone proteins<sup>5,6,8</sup>. Conversion reactions between purified PrP isoforms also have been stimulated by sulphated glycans and/or elevated temperature in the absence of denaturants<sup>9</sup>. Of intermediate complexity are conversion reactions between membrane-bound forms of PrP-sen and PrP-res<sup>10,64</sup>. These cell-free reactions, as well as cellular systems in which the acute formation of PrP-res in intact cells is monitored after exposure to PrP<sup>Sc</sup>-containing brain extracts<sup>11,12</sup> provide new experimental models of the cell-to-cell propagation of PrP<sup>Sc</sup> and infection. Conversion reactions have also been demonstrated in intact scrapie-infected brain slices<sup>13</sup>, cellular extracts<sup>12,14</sup>, and brain homogenates<sup>15</sup>. 

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**Prions for physicians**

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In the face of uncertainties about the exact nature of the infectious agent (prion) and the mechanism of its replication, one important motivation for PrP conversion studies is to determine whether or not some sort of ‘conversion’ of PrP\textsuperscript{C} alone or in combination with other defined factors, results in the generation of new TSE infectivity for animals. Unfortunately, in most of the above studies, the yield of PrP-res conversion product has been limited to amounts lower than that of the already infectious PrP-res used to induce the reaction, making it technically difficult to detect an increase in infectivity titre by bioassay in animals. One strategy for circumventing this problem has been to use a strain of PrP-res starter (or ‘seed’) from one species (e.g. hamster 263K scrapie) that does not itself induce illness in another species (e.g. mice), and perform interspecies conversion reactions using chimeric PrP-sen molecules that should be compatible with both species\textsuperscript{16}. In this scenario, any newly generated chimeric PrP-res should be capable of making the recipient species (mice) sick if PrP-res were, in fact, the infectious prion. Unfortunately, attempts to demonstrate the generation of new infectivity under a variety of conditions have failed in our laboratory (unpublished results) and in another\textsuperscript{17}.

In an effort to amplify PrP-res substantially in an \textit{in vitro} conversion reaction, Saborio and Soto developed the protein misfolding cyclic amplification (PMCA) system\textsuperscript{15}. In this system, detergent extracts of TSE-infected brain homogenate are mixed with vast excesses of similar extracts of PrP\textsuperscript{C}-containing normal brain tissue and subjected to repeated cycles of sonication and incubation. More than 30-fold increases in the amount of PrP-res over that provided in the infected brain extract were reported. This result suggested immediately that this procedure might be exploited to enhance the detection of PrP-res in TSE diagnostic tests. In addition, because the reported yield of newly formed PrP-res in this crude system was much higher than the yields observed in previously described conversion reactions between purified PrP isoforms, Saborio and Soto suggested that unidentified auxiliary factors provided in the crude normal-brain homogenate might be important. The PMCA technique should also provide opportunities to test whether or not the newly formed PrP-res is infectious; however, no such data have been published. In any case, the complexity of the whole-brain extracts used in the current PMCA PrP-res amplification scheme will leave open the question of whether factors besides PrP are critical in the composition and/or formation of TSE infectivity. Ultimately, in order to understand the nature of the TSE agent (prion) and its propagation fully, it will be necessary to bridge the gap between biochemically defined PrP conversion reactions, which so far have not proven to generate new TSE infectivity, and the TSE agent propagation that is known to occur readily in intact TSE-infected cells and animals.
Although the products of cell-free PrP conversion reactions have not been proven to be infectious, a correlation has been observed between the presence of infectivity and cell-free converting activity\(^\text{18}\). Furthermore, a wealth of data from transgenic mice and TSE-infected cell culture, suggest that interactions and molecular compatibility between PrP-res and PrP\(^\text{C}\) are important in the PrP-res formation and the transmission of TSE diseases (reviewed by Priola\(^\text{19}\) and Asante & Collinge\(^\text{20}\)). Hence, it remains important to define, at the molecular level, what occurs when the different PrP isoforms contact one another under various circumstances. The sections that follow consider: (i) the specificities and mechanism of the PrP-res/PrP-sen interactions; (ii) their apparent relationships to the control of interspecies TSE transmission; and (iii) the propagation of TSE strains.

**Sequence specificity of PrP-sen/PrP-res interactions and TSE transmissibilities**

When ‘heterologous’ cell-free conversion reactions have been performed using PrP-res of one species/sequence and PrP-sen of another, striking sequence specificity is often observed which seems to correlate with known susceptibilities of hosts to cross-species TSE infections (reviewed by Caughey *et al*\(^\text{21}\)). In comparing reactions driven by a given PrP-res preparation, 5- to > 50-fold stronger conversion efficiencies can occur with PrP-sen molecules from highly susceptible animals *versus* clearly resistant species/genotypes. Intermediate efficiencies (2–4-fold weaker than homologous) have been observed with PrP-sen from animals that are susceptible, but apparently less so than the original host species. Considering the limited information available, it seems that the log of the relative intracerebral transmission titre might be roughly proportional to the relative cell-free conversion efficiency\(^\text{22}\). However, much more quantitative transmission data between various species will be required to establish the fit between these parameters. Nonetheless, the requirement for molecular compatibility between different PrP-res and PrP-sen sequences, as reflected both *in vivo* and *in vitro*, appears to be an important factor in the transmission process.

In the absence of *in vivo* transmission data, cell-free conversion assays have been used tentatively to gauge the relative susceptibilities of various hosts to TSE agents from different source species or genotypes\(^\text{22–24}\). For instance, little is known about the transmissibility of CWD of deer and elk to non-cervid species\(^\text{25,26}\). In cell-free conversion reactions, CWD-associated PrP-res (PrP\(^\text{CWD}\)) of cervids readily induces the conversion of cervid PrP-sen molecules to the protease-resistant state consistent with the known transmissibility of CWD between cervids\(^\text{22}\). In contrast,
PrP\textsuperscript{CWD}–induced conversions of human, bovine and ovine PrP-sen were 14–100-fold, 5–12-fold, and 2–3-fold less efficient, respectively, than the most convertible cervid PrP-sen. These results demonstrate a barrier at the molecular level that presumably limits, but may not eliminate, the susceptibility of these non-cervid species to CWD. In addition, the data suggest that the rank order of susceptibilities to CWD, would be humans < cattle < sheep < cervids. Nonetheless, it is important to emphasize that these interspecies in vitro conversion experiments, while relatively rapid, are only weak surrogates for in vivo transmission and epidemiological data and should not be used as concrete evidence of species susceptibility or lack thereof.

Studies of the species specificity of conversion reaction have revealed that, in some interspecies combinations, the binding of PrP-sen to heterologous PrP\textsuperscript{Sc} can occur much more efficiently than the subsequent conversion to PrP-res\textsuperscript{7}. Thus, the species specificity of PrP-res formation may be determined more by the conversion step than the initial binding step. However, the binding of heterologous, non-converting PrP-sen molecules to PrP\textsuperscript{Sc} can interfere with the conversion of homologous PrP-sen, arguing that the convertible and non-convertible PrP-sen molecules compete for the same site. Such interference effects could explain reductions in the rates of PrP-res formation and/or TSE pathogenesis in hosts that co-express different PrP\textsuperscript{C} molecules such as humans who are heterozygous at PrP codon 129\textsuperscript{27,28}. Studies using mouse/hamster chimeric PrP have shown that the central part of the PrP-sen molecule, which encompasses three amino acid substitutions at mouse/hamster residues 138/139, 154/155, and 169/170, is important in the conversion of PrP-sen to PrP-res\textsuperscript{27,28}. Hence, critical interactions in the vicinity of these residues on PrP-sen and/or PrP\textsuperscript{Sc} may occur as part of the conversion step.

## Strain specificity of PrP conversion

Various TSE strains are associated with differences in PrP-res glycoform ratios, conformations, degrees of protease-resistance and aggregation states (reviewed by Horiuchi & Caughey\textsuperscript{29}). The mechanism by which these strain-dependent characteristics of PrP-res are maintained within individual host species in the absence of PrP sequence variation remains unclear. However, in vitro conversion reactions with largely purified PrP molecules have shown that different PrP\textsuperscript{Sc} ‘strains’ can impose their different conformations on a single species of unglycosylated PrP-sen. This provides evidence that the strain-specific conformers of PrP-res are faithfully propagated (or templated) through direct PrP\textsuperscript{C}–PrP-res interactions\textsuperscript{30}. Vorberg and Priola have demonstrated that different
strains of mouse PrPSc can also convert different PrPC glycoforms from the complex pool of glycosylated PrP molecules that are produced by cells. These observations suggest that because the pool of PrPC glycoforms can vary between cell types and within subcellular compartments, the cellular and subcellular sites of conversion can modulate the repertoire of conversion products. Thus, it is likely that the propagation of TSE strain-dependent forms of PrP-res is determined by the existence of multiple, self-propagating, and perhaps mutually exclusive, conformations or ordered aggregation states of PrP-res that preferentially select certain PrPC glycoforms from various cellular pools. There is also the possibility that the binding of other ligands such as metal ions and sulphated glycosaminoglycans might add to strain-associated PrP-res diversity. For instance, copper and zinc ions have been shown to alter the site at which PrP-res is cleaved in vitro by proteinase K. The complex interplay between PrP-res conformation, glycoform selection and ligand binding may cause PrP-res strains to target and be most efficiently propagated within subsets of cells that can provide the preferred glycoforms and ligands. This cellular targeting, as well as differences in susceptibility of target cells to the toxic effects of PrPSc accumulation may, in turn, establish strain-dependent patterns of pathological lesions in the central nervous system.

**Mechanism of conversion**

In the absence of knowledge of the three-dimensional structure of PrP-res and reconstitution of the minimal essential conditions for continuous conversion, it is impossible to describe fully the PrP conversion mechanism. Nonetheless, mechanistic studies have shown that the PrP conversion is induced by PrPSc aggregates/polymers and not soluble, monomeric forms of PrP and that newly converted PrP molecules become bound to the polymers. The greatly increased β-sheet content in PrP-res, as well as the fact that conversion can be stimulated by a variety of agents that affect protein conformation, suggest that the conversion involves a conformational change in addition to the binding of PrPC to PrP-res. PrP-sen first binds to PrP-res via a localized site near in space to the C-terminal end of the third helix and then is more slowly converted to the protease-resistant state. Modelling studies predicted a pre-eminent role of helix 1 of PrPC in the conformational change. Experiments have since shown that mutations of the helix 1 aspartates (D144 and D147) to neutral residues, which eliminate the possibility of stabilization by intrahelix salt bridges, can enhance conversion efficiencies by several fold. Although it has been postulated that...
conversion involves breakage of the single PrP<sub>C</sub> disulphide bond and formation of intermolecular disulphide-linked polymers<sup>38</sup> or domain-swapped dimers<sup>39</sup>, recent cell-free conversion reactions provide evidence that this is not the case<sup>40</sup>. Collectively, these and various other observations, such as the formation of amyloid fibrils by PrP-res, are consistent with an autocatalytic, templated or seeded polymerization mechanism (reviewed by Caughey <i>et al</i><sup>21</sup>).

Seeded protein polymerization mechanisms are well-established for yeast prion propagation and conventional amyloid fibril formation by other proteins and peptides<sup>21,41,42</sup>. Interestingly, abnormal protein aggregation is a key feature of a number of other neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease, Huntington’s disease and amyelotrophic lateral sclerosis. Concerning TSEs and these other neurodegenerative diseases, there are lively debates as to whether protein aggregation is a cause or an effect of the disease process, and what types of aggregates, if any, are most relevant in pathogenesis. With TSE diseases, the preponderance of experimental evidence supports the idea that salient properties of PrP<sub>Sc</sub> (<i>i.e.</i> partial protease-resistance, high β-sheet content, association with infectivity, and ability to cause PrP-sen to convert to PrP-res) are dependent upon its being in an oligomeric state (reviewed by Caughey <i>et al</i><sup>21</sup>). Full dissociation of PrP<sub>Sc</sub> usually, if not always, results in the irreversible loss of these properties. PrP<sub>Sc</sub> aggregates tend to be quite stable <i>in vitro</i> as indicated by the lack of dissociation of PrP monomers from PrP<sub>Sc</sub> aggregates<sup>35</sup>.

In PrP-res formation, as in well-characterized seeded protein polymerizations, there are presumably two phases<sup>43,44</sup>. The spontaneous formation of PrP-res from mutant PrP<sub>C</sub> is probably an important rate-determining step in familial TSE diseases. This step is analogous to a nucleation phase, which requires a rare or slow association of monomers to form a stable nucleus or seed. In TSEs of infectious origin, this step is bypassed altogether because of the introduction of PrP-res from an outside source. The most relevant mechanism in this case would then be PrP-res-induced conversion of PrP<sub>C</sub>, analogous to the growth phase of seeded polymerizations.

**Membrane associations and PrP conversion**

Although seeded polymerizations are often visualized in terms of linear fibrils, in the case of PrP-res formation it remains possible that the predominant mechanism involves a non-fibrillar autocatalytic assembly of GPI-anchored PrP molecules within the two-dimensional plane of membranes<sup>45</sup>. Interestingly, two-dimensional sheet-like arrays of PrP<sub>Sc</sub>
have been observed in addition to fibrils in purified preparations of PrPSc, and it seems that such arrays, or short, laterally bundled proto-fibrils, might also be possible on membranes. Alternatively, it is possible that the membrane environment somehow stabilizes a monomeric form of PrPSc; however, such a species has not been documented. Studies of the conversion reaction between GPI-anchored, membrane bound PrP isoforms revealed that conversion is not efficient when PrP-sen and PrPSc are attached to separate membrane vesicles and can only be detected when the vesicles are fused or PrPc is detached from its GPI anchor. On the other hand, if the PrP-sen is bound to membrane vesicles by a GPI-independent mode, then it is susceptible to conversion by PrPSc in separate vesicles. These observations not only provide evidence that PrP conversion can occur within the context of membrane, but also suggest that, in the process of infecting new cells, exogenous PrPSc must somehow be inserted into recipient cells’ membranes in a manner that allows the necessary contacts to be made between PrPSc and PrPc molecules. Consistent with this idea is evidence that contact between PrPSc and PrPc is made via surfaces that are close in space to the C-terminal GPI-anchor in the folded PrPc structure. Access to such surfaces by exogenous PrP molecules presumably would be limited by GPI anchoring to membranes.

**Potential co-factors**

Considering the difficulties in fully reconstituting continuous PrP conversion reactions with the purified PrP isoforms and the greater continuity of the PMCA reaction, it is tempting to conclude that there may be important conversion co-factors that remain poorly understood. The fact that PrP isoforms can bind sulphated glycosaminoglycans (GAGs) (Brimacombe et al. and references therein), glucose polymers, metal ions, lipids, nucleic acids, laminin, laminin receptor, protocadherin-2 and Bcl-2 (reviewed by Gauczynski et al.) makes them worthy of consideration as co-factors in PrP conversion. The influence of GAGs appears to be somewhat paradoxical since exogenous GAGs and GAG analogues can inhibit PrP-res formation in scrapie-infected cells and animals (see Caughey & Raymond and references therein), but can stimulate PrP-res formation in cell-free conversion reactions. In light of these findings, we have proposed that endogenous GAGs are important in PrP-res formation in vivo and that exogenous GAG-like inhibitors interfere with necessary interactions between PrP isoforms and the endogenous GAG co-factor.
Inhibitors of conversion as TSE therapeutic agents

Most of the compounds that are known to have anti-scrapie activity in animals are also known to be effective inhibitors of PrP-res formation in scrapie-infected neuroblastoma cells$^{31-54}$. Some, such as Congo Red and the porphyrins and phthalocyanines were first identified as potential anti-scrapie drugs in scrapie-infected tissue culture cells$^{31,32,55,56}$. Other classes of compounds such as synthetic PrP peptide fragments$^{57,58}$, lysosomotropic amines (e.g., quinacrine)$^{59}$, cysteine protease inhibitors$^{59}$, branched polyamines$^{60}$, cationic lipopolyamines$^{61}$, and mimics of dominant negative inhibitors of scrapie replication$^{62}$ have been shown to have anti-scrapie activity in this system, but have not been tested in animals. The track record so far indicates that inhibitors of PrP-res formation in vitro often have anti-TSE activity in vivo. In order to facilitate the screening of compound libraries for inhibitors of PrP-res formation and potential anti-TSE drugs, we and others$^{56,63}$ have pursued the development of higher throughput screens for inhibitors of PrP-res formation. Dot blot assays of PrP-res accumulation in scrapie-infected neuroblastoma cell cultures grown in a 96-well plate format have enabled us to screen hundreds of compounds per week (D Kocisko, L Raymond and B Caughey, unpublished results). Multi-well plate-based solid phase conversion assays can also facilitate the rapid testing of direct effects of compounds on PrP conversion (L Maxson, G Baron, and B Caughey, unpublished results). Potent and non-cytotoxic inhibitors that are identified in these screens are then tested for prophylactic and therapeutic efficacy against scrapie in rodents. Ultimately, it will be of particular interest to identify inhibitors that can cross the blood–brain barrier to potentiate therapeutic activity after TSE infections have spread to the central nervous system.

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