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Review

Role of lipid in forming an infectious prion?

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The infectious agent of the transmissible spongiform encephalopathies, or prion diseases, has been the center of intense debate for decades. Years of studies have provided overwhelming evidence to support the prion hypothesis that posits a protein conformal infectious agent is responsible for the transmissibility of the disease. The recent studies that generate prion infectivity with purified bacterially expressed recombinant prion protein not only provides convincing evidence supporting the core of the prion hypothesis, that a pathogenic conformer of host prion protein is able to seed the conversion of its normal counterpart to the likeness of itself resulting in the replication of the pathogenic conformer and occurrence of disease, they also indicate the importance of cofactors, particularly lipid or lipid-like molecules, in forming the protein conformation-based infectious agent. This article reviews the literature regarding the chemical nature of the infectious agent and the potential contribution from lipid molecules to prion infectivity, and discusses the important remaining questions in this research area.

Keywords prion; prion infectivity; prion protein conversion; lipid; recombinant prion

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Introduction

Transmissible spongiform encephalopathies (TSEs), also known as prion diseases, are a group of neurodegenerative disorders that affect humans and a wide variety of animals [1–6]. TSE research started with searching for the infectious agent in scrapie, the prototypic prion disease in sheep and goats [1,7–12]. Decades of studies have provided unequivocal evidence that the 'prion', a proteinaceous infectious particle, is responsible for the infectivity (reviewed in [1,13–22]). According to the prion hypothesis, PrP^{Sc}, the pathogenic conformer of host prion protein (PrP) and the principle constituent of prion, is capable of imprinting its aberrant conformation to the normal cellular PrP (PrP^{C}), resulting in the replication of PrP^{Sc} conformer and neurodegeneration [14,23]. The journey of exploring the chemical nature of the scrapie agent has been full of debates and controversies, along which numerous hypotheses have emerged and faded away [7,24–28]. The prion hypothesis, however, has withstood years of questioning and debate, and the latest studies have provided strong evidence supporting that the TSE agent is indeed a protein conformation-based infectious agent [29–41]. Despite these remarkable achievements, the molecular mechanism of prion infectivity, including the three-dimensional structure of the infectious PrP^{Sc}, the role of non-PrP cofactors in prion infectivity, and the molecular basis for PrP^{Sc} seeded conformational change of PrP^{C}, remains unclear. Here, we review the literatures regarding the chemical nature of the infectious agent in TSEs with a special emphasis on the potential contribution from lipid to prion infectivity.

Early Studies on Scrapie

Scrapie is the prototypic TSE that affects sheep and goat. As early as in mid-18th century, scrapie has been suggested as an infectious disease and detailed clinical manifestations were described [42]. In 1898, scientists first reported neuronal vacuolation as the characteristic neuropathological change in the brain of scrapie affected sheep [43]. However, the same group of scientists reported in the following year their failed attempts to transmit scrapie to healthy sheep [44]. Those negative results were largely due to the short observation period whereas the incubation time of scrapie could be extraordinarily long. Indeed, the first successful transmission of scrapie to healthy sheep was achieved after long incubation time (>14 months) [45]. Later, it was also demonstrated that scrapie could be experimentally transmitted to goats despite long incubation time (>15 months) [46,47]. Those studies clearly established that scrapie is an infectious disease.

The observations that the scrapie agent was able to pass through antibacterial filter [48] and scrapie infected animals developed diseases only after long incubation periods [45,46] led to the belief that scrapie was caused by an unidentified 'slow virus' [24]. Efforts to isolate the scrapie

agent failed to identify such 'virus', yet revealed the unusual physicochemical properties of the scrapie agent. In different laboratories, the scrapie agent was shown to survive diverse treatments that were used to inactivate viruses, including formalin treatment [49–51], boiling in water [52,53], extractions by low concentrations of phenol, chloroform [51] or ether [54,55], enzymatic degradation by nucleases [56–58], and ultraviolet (UV) and ionizing radiations [59,60]. Because of these unusual physicochemical characteristics, various speculations regarding the chemical nature of the scrapie agent emerged, including a protein [27], a polysaccharide [25,26], or a membrane fragment [7].

Inactivation of the Scrapie Agent by Ionizing and Ultraviolet Radiations

Protein-only theory

In an attempt to determine the size of the scrapie agent by ionizing radiation, Alper *et al*. [59] observed that an exceptionally high electron dose was required to inactivate the infectivity, leading to the prediction that the size of the scrapie agent was about 1/10 that of bacteriophages, the smallest virus at that time, and proposed for the first time that scrapie agent might replicate in the absence of nucleic acids. Based on the radiation inactivation result and other observations, Pattison and Jones [27] posited that the scrapie agent might just be a protein. To accommodate such self-propagating protein, Griffith [28] proposed three models to explain how a protein can replicate without nucleic acids. The second model, derived from a series of thermodynamic equations, predicts that the infectious agent (α_2), a protein dimer, could convert the normal cellular protein bearing a different conformation (α') into more protein dimers (α_2). This hypothetical mechanism is very similar to the currently accepted replication mechanism of scrapie agent: misfolded PrP^{Sc} induces the conversion of normal cellular PrP^{C} to PrP^{Sc}, a phenomenon now known as 'prion replication' [1,13,14,23].

Membrane hypothesis

In a follow-up study by Alper *et al*. [60], the scrapie agent was found to strongly resist inactivation by UV irradiation at the wavelength 254–265 nm, the wavelength where nucleic acids have the highest absorption and are most sensitive to inactivation. Inspired by the inactivation findings along with other distinct features of the agent, Gibbons and Hunter [7] proposed in the 'membrane hypothesis' that the scrapie agent might be a membrane fragment with a unique three-dimensional configuration. This unique membrane structure was hypothesized to self-replicate either by rearrangement of the sugar or polysaccharide molecules attached to the naïve membranes or through the self-replication of polysaccharides [25,26] on the surface of the naïve membranes. The involvement of lipid membrane in scrapie agent is more convincingly supported by several later studies conducted by Alper *et al*. [61,62]. In an inactivation study using near monochromatic UV light, it was shown that 237 nm is the most effective wavelength to inactivate scrapie agent and the inactivation at this wavelength is six times more efficient compared with inactivation at 254 or 280 nm [61]. Of note, most DNA and RNA viruses are more susceptible to UV irradiation at 254 nm and the inactivation spectrum for scrapie agent is obviously different from that of viruses, but very similar to the absorbance spectrum of the purified bacterial endotoxin (lipopolysaccharide), a glycolipid molecule [61]. The notion that lipids might be essential to scrapie infectivity was strongly supported by another inactivation study using ionizing radiation in an oxygenated aqueous solution [62]. Oxygen in aqueous suspensions protects bacteriophages, DNA, RNA, or protein molecules from ionizing radiation, but is extremely effective in destroying lipid membranes and scrapie infectivity [62], suggesting that lipids might be an essential component of the scrapie agent. On the basis of these findings, Alper [63] proposed a modified membrane hypothesis that incorporates PrP as part of the infectious agent.

Prion Hypothesis

The difficulties encountered in early attempts to purify the scrapie agent [54,57,58,64–67] fostered the debates surrounding the chemical nature of the agent. Employing a series of differential centrifugations, detergent extractions, and enzymatic degradations, Prusiner *et al*. [68,69] were able to isolate and purify the scrapie infectivity-enriched, partially protease-resistant protein species of 27–30 kDa, and in 1982 Prusiner [13] coined the term 'prion' as the infectious agent in scrapie, which was originally defined as 'small proteinaceous infectious particles that are resistant to inactivation by most procedures that modify nucleic acids'. The PrP of 27–30 kDa, or PrP27-30, was later identified as the N-terminus truncated, protease resistant core of PrP^{Sc}, the disease-associated conformational isoform of the normal cellular PrP^{C} [70–75]. The prion hypothesis postulates that the prion replication involves PrP^{Sc}-seeded conversion of PrP^{C} to PrP^{Sc} along with propagation of scrapie infectivity [1,14,23].

Although the possibility of large membrane fragments being part of the scrapie agent attenuated after prion was confirmed as the major component of the scrapie agent, the possibility that lipid molecules, regardless of the form and amount, might be involved in prion infectivity still remains [76–81].

In vitro conversion of PrP with denaturant treatment

Biophysical studies have shown that PrP^{Sc} is highly β-sheeted while PrP^{C} is mainly α-helical [82–85]. Owing to substantial conformational disparities between PrP^{C} and

PrP^{Sc}, a large energy barrier has to be overcome or lowered before the conversion could occur. Therefore, during the course of conversion to PrP^{Sc}, the folded PrP^{C} needs to be at least partially unfolded to reach a PrP* conformational state to make it kinetically feasible for the conversion (**Fig. 1**). Low concentrations of denaturants, including denaturing detergents or chaotropic agents, have been commonly used to partially denature PrP *in vitro*. In the first successful attempt to demonstrate PrP^{Sc}-seeded PrP^{C} conversion in a cell-free system, Kocisko *et al*. [86] showed that mixing partially denatured PrP^{Sc} and PrP^{C} together allowed the S35-labeled PrP^{C} to gain the signature PK-resistant conformation and co-aggregate with the unlabeled PrP^{Sc} seed. This cell-free conversion system not only for the first time demonstrated the seeding capability of PrP^{Sc}, but also further recapitulated the species barrier and prion strain phenomena *in vitro* [87,88]. However, the low efficiency of this system made it impossible to estimate the infectivity of newly formed S35-labeled PrP^{Sc}.

The successful purification of fully folded bacterially expressed recombinant PrP (recPrP) [89–92] and the remarkable structural similarity between recPrP and purified native PrP^{C} [93] make recPrP a legitimate substitute for PrP^{C} in cell-free conversion studies. In a low pH solution containing 1 M GdnHCl, Swietnicki *et al*. [94] converted the α-helical recPrP into high β-sheeted conformers, some of which could aggregate and form fibrils. Other studies revealed that the recPrP amyloid fibers could be formed much more efficiently with higher concentrations of denaturants and constant shaking [33,95,96]. The recPrP in the form of amyloid fibers, however, does not have the typical biochemical property of most PrP^{Sc} conformer, a strong C-terminal PK-resistance. Moreover, when the recPrP amyloid fibers formed by the latter approach were tested for prion infectivity in mouse bioassay, they were only able to induce disease in PrP overexpressing transgenic mice after a prolonged incubation period, but not in wild-type mice [33,97].

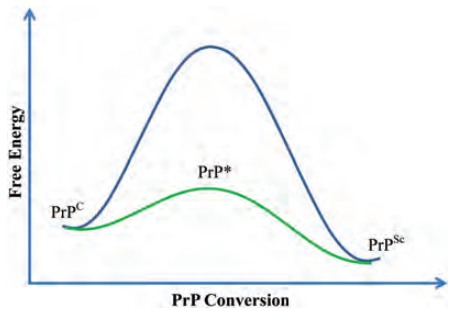

Figure 1 Diagram for PrP conversion In the presence of denaturants, lipid, or other co-factors, PrP^{C} can reach the PrP* state and further convert to PrP^{Sc}.

In vitro conversion of PrP with lipids

The low infectivity associated with recPrP amyloid fibers indicates that the denaturant treatment induced recPrP amyloid conformation is most likely quite different from the infectious PrP^{Sc} conformation. A better approach to unfold PrP and lower the energy barrier is probably required to generate the infectious PrP^{Sc} conformer. The observations from early studies mentioned above and the studies described in the following paragraph suggest to us that lipids may play such a role to facilitate PrP conversion.

The normal PrP^{C} is a cell surface glycoprotein attached to cell membrane via a glycosylphosphatidylinositol anchor and is released from lipid membranes after phosphoinositide phospholipase C (PI-PLC) digestion [98–101]. In contrast, PrP^{Sc} was tightly associated with lipid membranes and resistant to PI-PLC release, suggesting that the conversion from PrP^{C} to PrP^{Sc} might take place on the cell surface or endocytic pathway [100–102] and involves interactions between PrP and lipid membranes. In an improved cell-free conversion system that excludes the usage of denaturant and detergent, a direct interaction between PrP and lipid membranes was found to be essential for PrP conversion [103,104]. Using human recPrP and liposomes consisting of synthetic lipids, Morillas *et al*. [105] showed that the interaction between recPrP and anionic lipids destabilizes the C-terminal structured domain of PrP, which indicates that lipid interaction may unfold the PrP to reach PrP* state and facilitate its conversion to the infectious PrP^{Sc} conformation. Studies from Pinheiro group [106–109] also showed that binding to lipid membrane could lead to secondary structural changes, aggregation and fibrillization of hamster recPrP (90–231).

We carried out detailed studies on the interactions between full-length, α-helical-rich mouse recPrP and anionic lipids under physiologically relevant conditions (150 mM NaCl, pH 7.4) [110]. Following our finding that recPrP could hydrophobically interact with liposomes containing lipids extracted from mouse brain [111], we further showed that the recPrP–lipid interaction was initiated by the electrostatic binding between positively charged amino acid residues and negatively charged anionic lipid headgroups, which was followed by the hydrophobic interactions between recPrP hydrophobic domain and lipid acyl chains [110]. Such recPrP–lipid interactions could lead to a striking conformational change of recPrP, converting from the α-helical structure to a β-sheeted conformation accompanied by gaining of a C-terminal PK-resistance [110], both of which are the signature biochemical properties of PrP^{Sc} conformer [82–84]. Furthermore, we also showed that the recPrP–anionic lipid interaction does not ubiquitously lead to the PK-resistant recPrP conformations, and the resulting recPrP conformations were greatly influenced by the structure of phospholipid headgroups and the distribution of

these headgroups on the surface of lipid membranes. Another interesting observation from our study was that, depending on the composition of lipid membranes and the recPrP aggregation status, recPrP may also gain the N-terminal PK-resistance upon binding to lipid membranes [110]. Those results suggest that lipid membranes have a profound influence on PrP structure and PrP–lipid interactions may result in a variety of PrP conformations.

In the follow-up investigation, a series of recPrP mutations, including deletion of the N-terminal positively charged region, deletion of the highly conserved hydrophobic region, and point mutations in the middle charged regions, were employed to dissect the recPrP–lipid interactions [112]. Deletion of the conserved middle hydrophobic region completely demolished the lipid–recPrP hydrophobic interaction and resulted in an absolute loss of the PK-resistance, supporting a key role of hydrophobic PrP–lipid interaction in generating PK-resistant conformation. Deletion of the N-terminal positively charged region was found to reduce electrostatic interaction between recPrP mutant and anionic lipid, but the effect is not strong enough to alter the hydrophobic interaction and consequent PK-resistance. In contrast, mutations that eliminate the positive charges in front of the highly conserved hydrophobic region in the middle part of PrP appeared to affect neither electrostatic nor hydrophobic recPrP–lipid interaction, but markedly reduced the PK-resistance, suggesting a role of the middle charged residues in orienting recPrP and assisting the formation of PK-resistance. More interestingly, despite the similarity in location and amino acid substitution, the two human prion disease-associated mutations, P102L and P105L, exhibited dramatically different effects on recPrP–lipid interactions. The P102L mutant did not have an apparent effect on either electrostatic or hydrophobic PrP–lipid interaction, but completely abolished the PK-resistance. The P105L mutant, however, had a more complicated influence on recPrP–lipid interactions. Although the P105L mutation did not change the net charge of PrP, it significantly reduced the electrostatic PrP–lipid interaction. Furthermore, it had no apparent effect on the hydrophobic PrP–lipid interaction, but markedly changed the PK-resistance pattern. Since both mutations are located in the middle charged region and flanked by charged lysine residues, these results support a role of the middle charged region in the formation of PK-resistant PrP conformations. Moreover, the dramatic influence of both disease-associated mutations on PrP–lipid interaction and the resulting PrP conformations supports a role of lipid–PrP interaction in the pathogenesis of prion disease.

These results clearly showed that the recPrP–lipid interaction is sufficient to convert α-helical-rich recPrP to the β-sheeted, PK-resistant PrP^{Sc}-like conformations under physiologically relevant conditions, which supports that, similar to denaturant treatment, the lipid interaction is able to unfold recPrP and lower the energy barrier for converting recPrP to a conformation similar to PrP^{Sc} (**Fig. 1**).

In vitro generation of infectious prions

Although most of the foregoing conversion products recapitulate biochemical features of PrP^{Sc}, such as aggregation, β-sheeted secondary structure and protease resistance, none of which has been shown to induce prion disease in wild-type animals [33,97,113]. To support the prion hypothesis, demonstrating *bona fide* prion infectivity with converted PrP species is critically needed.

On the basis of the idea that fragmentation of protein aggregates by sonication increases the seeding capability, Soto *et al*. [114–116] developed a new technique termed Protein Misfolding Cylic Amplification (PMCA), in which the PrP^{Sc}-containing diseased brain homogenate and normal hamster brain homogenate with excessive PrP^{C} were mixed in a testing tube and subjected to rounds of alternating sonication and incubation. The development of PMCA technique represents a landmark advance in prion research, which has been demonstrated to efficiently propagate PrP^{Sc} derived from various sources [35,117–119]. More importantly, the prion infectivity was concomitantly propagated with the PrP^{Sc} conformation and resulted in *bona fide* prion disease in wild-type hamsters [35]. Further studies have revealed that prion species barrier and prion strain phenomena could also be recapitulated by PMCA in a cell-free environment [120–122]. A major difference between PMCA and other cell-free conversion systems is the usage of brain homogenates and the non-PrP cofactors in the brain homogenates may facilitate the PrP conversion. Indeed, many studies have argued for the involvement of cofactors in PrP conversion, which includes polyanions such as proteoglycan [123] or RNA [124], metal ions [125–127] and lipids [103–110,112].

Proteoglycan was the first polyanion to show an effect in promoting the PrP^{Sc} propagation in a cell-free conversion system [123]. Nucleic acids, including both DNA and RNA, are able to interact with PrP and induce conformational changes of PrP [128,129] and RNA molecules have been found to be a potent cofactor fostering PrP^{Sc}-seeded *in vitro* prion propagation [124,130]. Using serial PMCA technique, Deleault *et al*. [36] successfully generated infectious hamster prion from purified native hamster PrP^{C} with co-purified lipid molecules and additional RNA molecules, further supporting that RNA is potent factor promoting the formation of PrP^{Sc} conformer.

Early inactivation studies by Alper have implicated lipid as an essential component of prion infectivity [61,62], which was also supported by several lines of evidence from other laboratories. It has been shown that even the purest preparations of prion rod contain at least two different types

of lipid molecules albeit in a very small amount [79]. Eluting solubilized prion rod through sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) dissociates PrP27-30 from lipids and the eluted PrP27-30 completely lost prion infectivity [80]. Altered PrP27-30 conformation resulting from SDS-PAGE may explain the loss of infectivity in the frame of 'protein only' hypothesis, but an alternative interpretation that lipid–PrP interaction is essential for maintaining the infectious conformation could stand up by its own as well. Consistent with the latter explanation, purified prion rods could be dissolved into liposomes without losing infectivity; indeed, the infectivity titer associated with those prion liposomes increased 10–100 folds as evaluated by either incubation time or end-point titration bioassays [76,77]. In accord with the finding is a recent study showing that microsome associated PrP^{Sc} could infect SN56 cells with an efficiency higher than purified PrP^{Sc} [81]. The increased infectivity has also been suggested to result from the disassociation of large PrP aggregates, which could produce higher concentration of smaller PrP oligomers [77,81], which have been shown to possess the highest infectivity [131]. Also being suggested is that the lipid membrane may mediate a faster distribution of PrP^{Sc} or PrP27-30 onto cell plasma membranes resulting in faster prion propagations [77,81]. Nonetheless, the enhanced infectivity of lipid associated PrP^{Sc} or PrP27-30 supports a role of lipid in prion infectivity.

Inspired by the foregoing findings and our observations that recPrP–lipid interaction induces recPrP conformational change and lead to a PK-resistant conformation similar to PrP^{Sc} [110], we tested the possibility of generating infectious prion via serial PMCA with bacterially expressed recPrP in the presence of two cofactors, the synthetic phospholipid POPG (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol)) and total RNA isolated from normal mouse liver [37]. The generation of a PK-resistant recPrP conformer (denoted as rPrP-res) was achieved after rounds of PMCA and the *in vitro* generated rPrP-res possesses all the hallmarks of diseased brain derived PrP^{Sc}: aggregated, C-terminal PK-resistant, capable of seeding the native PrP^{C} conversion by PMCA, and able to infect cultured cells [37]. Most importantly, the newly generated rPrP-res induced prion disease in wild-type mice after a relatively short incubation period and with a quite synchronized onset, characteristics of a highly infectious prion [37]. We have shown that highly infectious rPrP-res can be generated when total mouse liver RNA was replaced by synthetic polyriboadenylic acid [poly(rA)] in a later study [40], ruling out the possibility that RNA carries the genetic information necessary for infection [36,40].

Prion infectivity has also been generated with bacterially expressed recPrP using PrP^{Sc}-seeded PMCA in the presence SDS and triton [38]. The recPrP conformer produced by this approach is able to cause prion disease in wild-type hamsters, but with a rather large variability in the incubation time and attack rate. In addition, the recPrP amyloid fibers going through an 'annealing' step (five cycles of incubations at 80°C and 37°C in the presence of normal hamster brain homogenate or bovine serum albumin) induced prion infectivity in asymptomatic wild-type hamsters [39]. More recently, prion infectivity has been successfully propagated by PMCA using recPrP plus a sole cofactor, phosphoethanolamine (PE), and more interestingly, when three diverse prion strains were propagated to recPrP with PE as the sole cofactor, they appear to converge to a single new strain type [41,132], supporting the notion that polyanions are dispensable for prion infectivity [133] and suggesting a role of cofactor in modulating prion strain phenotype.

Collectively, all these studies have demonstrated that purified bacterially expressed recPrP is able to convert to the infectious conformers in various *in vitro* systems, providing unequivocal evidence supporting the prion hypothesis.

Closing Remarks

Significant progress has been made in understanding the chemical nature of the infectious agent in TSEs, but several important questions regarding the agent still remain to be answered. Although an important role of lipids in prion infectivity is supported by the early radiation inactivation studies [61,62] and recent recombinant prion studies [37,41,134], whether lipids or lipid-like molecules are essential component of the infectious agent remains unclear. Further studies are also needed to clarify whether different types or combinations of cofactors [135], including both lipids and polyanions, contribute to prion strain diversity [4,136–138], the formation of seemingly endless PrP^{Sc} conformers [4,139,140], and the species barrier in prion transmission [141]. Moreover, the ability to generate recombinant prion in a clean *in vitro* system with defined synthetic cofactors opens the avenue to solve the three-dimensional structure of infectious PrP^{Sc} conformation, which is essential for us to understand the molecular mechanism of prion propagation. For this purpose, further optimization of the *in vitro* recombinant prion propagation system is needed to increase the quantity and quality of recombinant prion in order to meet the requirement of structural studies. Further investigations in this area are not only critical for unraveling the molecular mechanism of this unorthodox infectious agent and developing novel strategies against these fatal neurodegenerative disorders, they may also shed light on the mechanism of recently discovered 'prion-like' propagation of protein aggregates in a variety of common neurodegenerative disorders.

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