Review

Decipher the mechanisms of rabbit's low susceptibility to prion infection

Zhen Yuan, Deming Zhao, and Lifeng Yang*

State Key Laboratories for Agrobiotechnology, Key Lab of Animal Epidemiology and Zoonosis, Ministry of Agriculture, National Animal Transmissible Spongiform Encephalopathy Laboratory, College of Veterinary Medicine, China Agricultural University, Beijing 100193, China
*Correspondence address. Tel/Fax: +86-10-62732987; E-mail: yanglf@cau.edu.cn

Rabbits have low susceptibility to prion infection. Studies on prion protein (PrP) from animal species of different susceptibility to prion diseases identified key amino acid residues, specific motif, and special features in rabbit prion protein (RaPrP^C) that contribute to the stability of rabbit PrP^C and low susceptibility to prion infection. However, there is no evidence showing that rabbits are completely resistant to prion diseases. It has been reported that the rabbit prion could be generated *in vitro* through protein misfolding cyclic amplification and proved to be infectious and transmissible. Here, we reviewed studies on rabbit-specific PrP structures and features in relation to rabbit's low susceptibility to prion infection.

Keywords   prion diseases; rabbit; susceptibility; resistance; prion protein

Introduction

Since the transmissible spongiform encephalopathy (TSE) emerged around 1730 in France with the description of an uncommon disease in sheep named 'scrapie' [1], continuous researches into natural TSE cases have resulted in an increasing list of TSEs and species susceptible to this family of diseases. Currently known TSEs include Creutzfeldt–Jakob disease, Gerstmann–Sträussler–Scheinker syndrome, fatal familial insomnia and kuru in humans, scrapie in goats and sheep, bovine spongiform encephalopathy in cattle, and chronic wasting disease in elk and deer [2]. It has also come to light that some species have low susceptibility to clinical TSE [3]. Thus, animals could be divided into two groups according to their TSE susceptibility: highly susceptible group (including hamster, mice, cattle, sheep, and deer) and lowly susceptible group (including rabbit, horse, and dog) [4,5]. To understand species susceptibility to TSEs and the

mechanism of prion disease susceptibility, Gibbs and Gajdusek [5] made many attempts to infect rabbits with different prion strains, but all failed. Unsuccessful experimental challenges to infect rabbits with prion and lack of documented natural TSE cases in this species had led to the belief that rabbits have low susceptibility to TSEs.

Fernandez-Funez *et al*. [6] found *in vivo* evidence to support the decades-old observations on prion susceptibility by creating transgenic flies expressing wild-type, full-length hamster, mouse, or rabbit prion protein (PrP). They compared the transgenic flies expressing PrP of different species by biochemical, histological, and behavioral assays, and found that rabbit PrP did not induce neurodegeneration in the brains of transgenic flies, while hamster PrP could be misfolded into PrP^{Sc}-like conformers and induced strong vacuolar changes in brain neurons [7]. In order to directly test neuronal dysfunction, they assayed the ability of the three proteins to induce progressive locomotor dysfunction. Both hamster and mouse PrP induced locomotor defects in a few days, whereas flies infected with the rabbit PrP were similar to control flies. In addition, only hamster and mouse PrP were recognized by a conformational antibody specific for PrP^{Sc}-like structures [8]. The inability of rabbit PrP to acquire conformation to induce PrP pathobiology clearly distinguished rabbit PrP from hamster or mouse PrP. These studies supported the belief that rabbits have low susceptibility to TSEs.

According to the widely accepted 'protein only' hypothesis, an abnormal isoform of host-encoded cellular PrP (PrP^C) is the principal and possibly the sole constituent of the transmissible agent or prion [2,9,10]. The β-state is a monomeric folding intermediate that lay between the native and unfolded state in the PrP folding pathway [11], and is similar to β-structured oligomers of proteins involved in other neurodegenerative diseases. It plays an important role in the development of prion diseases [12]. Khan *et al*. [13] showed that the β-state PrP is highly toxic in cell culture. The propensity of PrP to form β-state PrP from hamsters, mice, rabbits, horses, and dogs was highly correlated with

the prion disease susceptibility of the species. Thus, the propensity to form β-state PrP was considered a valid marker of prion disease susceptibility [13], and could be used for the assessment of animal's susceptibility to prion diseases.

Several independent studies on rabbit PrP gained insights into the molecular principles regulating PrP conversion and stability, which may account for the low susceptibility of rabbits to prion diseases. We reviewed studies on the unique molecular structures and their relationship to the stability of rabbit PrP, which may be important determinants of rabbit's low susceptibility to prion diseases.

Resistance of Rabbit PrP to Denaturation

To understand the structural characteristics of PrP, Khan *et al.* [13] subjected recombinant PrP 90–231 of different susceptible animals including Syrian hamster, mouse, rabbit, horse, and dog to comparative biochemical studies. Using hamster PrP 90–231 at 0, 2.5, and 7.5 M urea (pH 4), respectively, with a two-wavelength method of circular dichroism (CD), they displayed a typical α-helical structure, a β-sheet-rich structure, and an unfolded structure. The β-sheet-rich structure denoting the β-state appeared under moderately acidic pH and denaturing conditions (pH 4; 2.5 M urea). Then, using CD, they measured the relative amount of β-state of hamster, mouse, rabbit, horse, and dog PrP under moderate to strong denaturing conditions (pH 7 to 4 and 0–7.5 M urea). At neutral pH (7.0), none of the proteins were in the β-state regardless of urea concentration, but with increasing acidic conditions they detected varying concentrations of the β-state. At pH 5.0, only hamster PrP was in the β-state. At pH 4.5, both hamster and mouse PrP accumulated a mass of β-state, while rabbit PrP formed a small fraction of β-state. At pH 4.0, all five species PrP had the β-state fraction. However, horse and dog PrP had smaller fractions of β-state than hamster, mouse, and rabbit, indicating that the propensity to form the β-state is the greatest for hamster PrP, followed by mouse PrP, and then by rabbit, horse, and dog PrP [13]. This propensity, to some degree, proves rabbit's low susceptibility to prion diseases in PrP structure, because it seems that rabbit PrP tolerated the denaturation by acid and urea more than hamster and mouse PrP, although rabbit PrP was more prone to the formation of the β-state than horse and dog PrP.

Amino Acid Residues Specific to Rabbit PrP

PrP molecules that include either rabbit- or mouse-specific amino acid sequences were constructed from the N-terminal region (residues 1–111), central region (residues 112–177), and C-terminal region (residues 178 –254) [14]. To find out whether amino acid residues within these three regions of the rabbit PrP molecule affected the formation of PrP^{Sc},

Vorberg *et al.* [14] made substitutions of mouse PrP amino acid sequence with rabbit PrP amino acid sequence. Substitution of mouse PrP amino acid sequence with rabbit PrP amino acid sequence at the C-terminus drastically decreased, but did not abolish, the formation of PrP^{Sc}. However, substitution of mouse amino acid sequence with rabbit amino acid sequence at the N-terminus totally prevented PrP^{Sc} formation. Conversely, substitution of the rabbit amino acid residues in the C-terminus with the mouse PrP residues failed to convert the rabbit PrP to a protease resistant form. These results suggested that amino acid residue differences within the N-terminus and C-terminus, and possibly the central portion of the rabbit PrP molecule all negatively affect the formation of PrP^{Sc}.

Mouse and rabbit PrP have 87% sequence identity, but there are 22 amino acids that are different, and several of these residues are in regions of PrP known to be important in PrP^{Sc} formation. In scrapie-infected mouse neuroblastoma cells, residues Gly99 and Met108 within the N-terminus, Ser173 within the central region, and Ile214 within the C-terminus of rabbit PrP were shown to inhibit PrP^{Sc} generation when incorporated into mouse PrP, suggesting that multiple amino acid residues in rabbit PrP inhibit PrP^{Sc} formation (**Fig. 1**). Unfortunately, the authors did not clarify how different amino acid residues inhibit PrP^{Sc} formation.

Nisbet *et al.* [15] found that approximately one-third of amino acid differences between mouse and rabbit PrP^{C}s were shown to occur at the glycosylphosphatidylinositol (GPI) anchor attachment site (ω site), and they predicted that the difference in amino acid sequence around the ω site could alter the GPI anchor of RaPrP hindering prion propagation. To investigate whether these residues confer rabbits' low susceptibility to prion infection, they created a mutant PrP model, mouse RK-13 cells expressing MoPrP-RbGPI. They found that these mutant RK cells were resistant to infectious prions and did not generate protease-resistant PrP. These results suggested that the rabbit-specific GPI anchor attachment site controls the conversion of RaPrP^C into PrP^{Sc} [15].

In addition, I214V and S173N substitutions were reported to result in distinct structural changes in RaPrP^C [16,17]. The above findings suggested that several amino acid residues may be implicated in the inability of RaPrP^C to convert into PrP^{Sc}, but the exact and key residues responsible for the low susceptibility of rabbits to prion diseases are yet to be clarified.

Hydrophobic Staple-like Helix-capping Motif in Rabbit PrP^C

Using the two-wavelength CD method with X-ray crystallography, Khan *et al.* [13] identified a key helix-capping motif that controls the formation of the β-state may govern

		99		108	
RaPrP	[90]GQGGGTHNQWGKPSKPKTSMKHVAGAAAAGAVVGGLGGYMLGSAMSRPLIHFGNDYEDRYYRENMYRYPNQVYYRPV[166]				
MoPrP	GQGGGTHSQWNKP SKPKTNLKHMAGAAAAGAVVGGLGGYVLGSAMSRP I IHFGSDYEDRYYRENMHRYPNQVYYRPM				

		173		214	
RaPrP	[167]DQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETD IK I MERVVEQMC I TQYQQESQAAYQRAAG[230]				
MoPrP	DQYSNQNNFVHDCVNITI KQHTVTTTTKGENFTETDVKMMERVVEQMCVTQYQKESQAYYDGRRS				

Figure 1 Sequence alignment of the mouse and rabbit PrP$^{90-230}$ constructs The distinct rabbit amino acids are colored blue. The red number 99, 108, 173, 214 rabbit amino acids were shown to inhibit PrP^{Sc} generation.

the susceptibility to prion diseases. In rabbit PrP^C 121–231, the backbone amide of S174 and the side-chain carbonyl of N171 form a hydrogen bond. The backbone carbonyl of N171 backwards binds to the side-chain hydroxyl group of S174. Thus, a helix-capping motif that is unique in rabbit PrP structure is formed through these reciprocal side-chain to backbone hydrogen bonds and the flanking hydrophobic residues Y169 and F175 [18,19]. To determine the function of this unique motif, the authors constructed a S174N mutant. The S174N mutation in this helix-capping motif allowed the mutated rabbit PrP^C to be in the β-state at pH 4.5 and 4 mM urea in which the wild-type rabbit PrP^C cannot be in the β-state. The disruption of this helix-capping motif increased the β-state propensity of rabbit PrP^C to levels similar to the PrP^C from mouse, a species more susceptible to prion diseases. These findings suggested that the helix-capping motif formed between residues 171 and 174 could be responsible for the conformational stability of rabbit PrP^C and rabbits' low susceptibility to prion diseases.

A Highly Ordered β2–α2 Loop

To understand the structural stability of rabbit PrP^C, Wen *et al.* [17] used multi-dimensional heteronuclear nuclear magnetic resonance (NMR) techniques to determine the structure of the recombinant protein RaPrP^C-(91–228) and its S173N variant. They used CD spectroscopy to assess the conformational stability upon urea-induced denaturation and performed 15N relaxation measurements to detect the backbone dynamics of their globular structured C-terminal domains (121–228). RaPrP^C was found to possess a unique charge distribution, carrying a continuous area of positive charges on the surface, which distinguishes RaPrP from other PrP^{C}s. Because intermolecular interactions are sensitive to surface charge, the unusual positive charges in rabbit PrP^C may inhibit prion conversion. The authors produced detailed data on the dynamics of rabbit PrP^C that point to the highly organized state of the β2–α2 loop [13] and they proposed that the highly ordered β2–α2 loop in rabbit PrP^C may contribute to the local as well as global stability of the protein. The NMR dynamics analysis revealed a distinct increase in the structural flexibility of loop 165–172

and helix-3 after the S173N substitution, implying that the S173N substitution disturbs the long-range interaction between loop 165–172 and helix-3, which further leads to a marked decrease in the global conformational stability. The S173N substitution caused visible changes of the charge distribution around the recognition sites. These results suggested that the ordered loop 165–172 and its interaction with helix-3, together with the unique distribution of surface electrostatic potential, significantly contribute to the unique structural characteristics of RaPrP^C.

Salt Bridges at D201–R155 and D177–R163

There always exist salt bridges between D202–R156 and D178–R164 in human and mouse PrPs, and between D201–R155 and D177–R163 for the rabbit PrP [20,21]. Zhang [22] compared the stability of mouse, rabbit, and human PrPs by molecular dynamics simulation. Salt bridges at D202–R156 and D178–R164 in human and mouse PrPs and at D201–R155 and D177–R163 in the rabbit PrP were removed under low pH conditions. Removing the salt bridges did not alter the secondary structures of human and mouse PrPs, while the helical structure of the wild-type rabbit PrP collapsed. Thus, it appears that the D177–R163 and D201–R155 salt bridges contribute to the structural stability of the rabbit PrP.

Amyloid Fibril Formation

Amyloid fibrils are associated with neurodegenerative diseases such as Alzheimer disease, prion diseases, and amyotrophic lateral sclerosis [23–26]. The formation of amyloid fibrils is thought to result from failures of cellular quality control mechanisms including molecular chaperones, proteolysis, autophagy, and proteasomes [27,28]. Zhou *et al.* [29] studied the effects of two 'inert' polymeric crowding agents, Ficoll 70 and dextran 70, on the kinetics of amyloid formation of the recombinant full-length rabbit, human, and bovine PrP using ThT binding assays. They showed that the macromolecular crowding agents had different effects on fibrillation of the recombinant PrPs of different species. While these agents dramatically promoted fibril formation of

human and bovine proteins, they appeared to significantly inhibit fibrillation of the rabbit protein. The above findings were consistent with the experimental results of Ma *et al*. [30], who also reported inhibition of fibril formation of rabbit PrP and enhancement of amyloid fibril formation of human and bovine PrP by the crowding agents. It was observed that amyloid fibrils formed by the rabbit protein contained more α-helix structure and less β-sheet structure than the human and bovine proteins [29]. Therefore, the inhibition of fibril formation of the rabbit protein in the crowded physiological environment could be one of the reasons why rabbits have low susceptibility to prion diseases.

Generation of Rabbit PrP^{Sc} *in vitro* through Protein Misfolding Cyclic Amplification

Chianini *et al*. [31] generated rabbit PrP^{res} *in vitro* by subjecting unseeded normal rabbit brain homogenate to serial-automated protein misfolding cyclic amplification (saPMCA). To test the infectivity and transmission of the rabbit PrP^{res}, they challenged intracerebrally 3-month-old male New Zealand white rabbits of identical PrP genotype with the generated PrP^{res}. After 766 days, one out of three challenged rabbits developed clinical neurological signs of transmissible spongiform encephalopathies and died 4 days later. Biochemical analysis of the dead rabbit's brain by western blot analysis confirmed the presence of PrP^{Sc}. Histological and immunohistochemical studies showed vacuolation of neurons and astrocytosis in the brain of the challenged rabbit. Furthermore, they challenged three 8-week-old male transgenic mice over-expressing rabbit PrP^{C} with 1% brain homogenate containing abnormal PrP prepared from the clinically affected rabbit. As a result, all three prion-inoculated mice started showing clinical signs of prion disease at about 266 days. Biochemical analysis of their brains confirmed the presence of PrP^{Sc}. They also challenged rabbits with brain homogenate from the clinically affected rabbits. Western blot analysis of brain homogenate from the dead and the diseased rabbits confirmed the presence of PrP^{Sc} in all of the brain areas examined. The above results showed that the rabbit prion generated *in vitro* are infectious and transmissible, and suggested that rabbits are not completely resistant to prion infection.

Conclusion

The PrP of rabbits have many special structures including specific amino acid residues, hydrophobic staple-like helix-capping motif, highly ordered β2−α2 loop, and salt bridges at D201−R155 and D177−R163. These special structures hinder rabbit PrP from forming amyloid fibril and β-state, and thus may fully account for the phenomenon that rabbits have low susceptibility to prion diseases. However, the rabbit prion can be generated *in vitro* through PMCA and

proved to be infectious and transmissible. Then, can we abandon the current notion of the low susceptibility of rabbits to prion diseases? The rabbit prion was only produced through PMCA *in vitro*, but not by challenging rabbits directly *in vivo* with other known prion strains. Thus, we cannot yet say that rabbits are susceptible to prion diseases.

Most importantly, the studies on special structures of rabbit PrP^{C} contributing to the stability of rabbit PrP^{C} give us valuable knowledge of conversion of PrP^{C} to the pathogenic form. Meanwhile, they also provide clues to design novel therapeutic approaches to block PrP conversion and disease propagation.

Funding

References

1 Deslys JP PA. Mad cow disease: the risks to humans. France: DOMINOS Flammarion 2001, 23−24.

2 Prusiner SB. Novel proteinaceous infectious particles cause scrapie. Science 1982, 216: 136−144.

3 Fernandez-Borges N, Chianini F, Erana H, Vidal E, Eaton SL, Pintado B and Finlayson J, *et al*. Naturally prion resistant mammals: a utopia? Prion 2012, 6: 425−429.

4 Barlow RM and Rennie JC. The fate of ME7 scrapie infection in rats, guinea-pigs and rabbits. Res Vet Sci 1976, 21: 110−111

5 Gibbs CJ, Jr and Gajdusek DC. Experimental subacute spongiform virus encephalopathies in primates and other laboratory animals. Science 1973, 182: 67−68.

6 Fernandez-Funez P, Zhang Y, Casas-Tinto S, Xiao X, Zou WQ and Rincon-Limas DE. Sequence-dependent prion protein misfolding and neurotoxicity. J Biol Chem 2010, 285: 36897−36908.

7 Fernandez-Funez P, Casas-Tinto S, Zhang Y, Gomez-Velazquez M, Morales-Garza MA, Cepeda-Nieto AC and Castilla J, *et al*. In vivo generation of neurotoxic prion protein: role for hsp70 in accumulation of misfolded isoforms. PLoS Genet 2009, 5: e1000507.

8 Fernandez-Funez P, Zhang Y, Sanchez-Garcia J, Jensen K, Zou WQ and Rincon-Limas DE. Pulling rabbits to reveal the secrets of the prion protein. Commun Integr Biol 2011, 4: 262−266.

9 Griffith JS. Self-replication and scrapie. Nature 1967, 215: 1043−1044.

10 McKinley MP, Bolton DC and Prusiner SB. A protease-resistant protein is a structural component of the scrapie prion. Cell 1983, 35: 57−62.

11 Hornemann S and Glockshuber R. A scrapie-like unfolding intermediate of the prion protein domain PrP(121−231) induced by acidic pH. Proc Natl Acad Sci USA 1998, 95: 6010−6014.

12 Collinge J and Clarke AR. A general model of prion strains and their pathogenicity. Science 2007, 318: 930−936.

13 Khan MQ, Sweeting B, Khipple Mulligana V, Eli Arslanb P and Cashmanc NR. Prion disease susceptibility is affected by β-structure folding propensity and local side-chain interactions in PrP. Proc Natl Acad Sci USA 2010, 107: 19808−19813.

14 Vorberg I, Groschup MH, Pfaff E and Priola SA. Multiple amino acid residues within the rabbit prion protein inhibit formation of its abnormal isoform. J Virol 2003, 77: 2003–2009.

15 Nisbet RM, Harrison CF, Lawson VA, Masters CL, Cappai R and Hill AF. Residues surrounding the glycosylphosphatidylinositol anchor attachment site of PrP modulate prion infection: insight from the resistance of rabbits to prion disease. J Virol 2010, 84: 6678–6686.

16 Wen Y, Li J, Xiong M, Peng Y, Yao W, Hong J and Lin D. Solution structure and dynamics of the I214V mutant of the rabbit prion protein. PLoS ONE 2010, 5: e13273.

17 Wen Y, Li J, Yao W, Xiong M, Hong J, Peng Y and Xiao G, *et al*. Unique structural characteristics of the rabbit prion protein. J Biol Chem 2010, 285: 31682–31693.

18 Aurora R and Rose GD. Helix capping. Protein Sci 1998, 7: 21–38.

19 Seale JW, Srinivasan R and Rose GD. Sequence determinants of the capping box, a stabilizing motif at the N-termini of alpha-helices. Protein Sci 1994, 3: 1741–1745.

20 Zhang J. Studies on the structural stability of rabbit prion probed by molecular dynamics simulations. J Biomol Struct Dyn 2009, 27: 159–162.

21 Zhang J. Studies on the structural stability of rabbit prion probed by molecular dynamics simulations of its wild-type and mutants. J Theor Biol 2010, 264: 119–122.

22 Zhang J. Comparison studies of the structural stability of rabbit prion protein with human and mouse prion proteins. J Theor Biol 2011, 269: 88–95.

23 Dobson CM. Protein folding and misfolding. Nature 2003, 426: 884–890.

24 Goedert M. Tau protein and the neurofibrillary pathology of Alzheimer's disease. Trends Neurosci 1993, 16: 460–465.

25 Pan KM, Baldwin M, Nguyen J, Gasset M, Serban A, Groth D and Mehlhorn I, *et al*. Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. Proc Natl Acad Sci USA 1993, 90: 10962–10966.

26 Polymenidou M and Cleveland DW. The seeds of neurodegeneration: prion-like spreading in ALS. Cell 2011, 147: 498–508.

27 Lee SJ, Desplats P, Sigurdson C, Tsigelny I and Masliah E. Cell-to-cell transmission of non-prion protein aggregates. Nat Rev Neurol 2010, 6: 702–706.

28 Wickner S, Maurizi MR and Gottesman S. Posttranslational quality control: folding, refolding, and degrading proteins. Science 1999, 286: 1888–1893.

29 Zhou Z, Yan X, Pan K, Chen J, Xie ZS, Xiao GF and Yang FQ, *et al*. Fibril formation of the rabbit/human/bovine prion proteins. Biophys J 2011, 101: 1483–1492.

30 Ma Q, Fan JB, Zhou Z, Zhou BR, Meng SR, Hu JY and Chen J, *et al*. The contrasting effect of macromolecular crowding on amyloid fibril formation. PLoS ONE 2012, 7: e36288.

31 Chianini F, Fernandez-Borges N, Vidal E, Gibbard L, Pintado B, de Castro J and Priola SA, *et al*. Rabbits are not resistant to prion infection. Proc Natl Acad Sci USA 2012, 109: 5080–5085.