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New Molecular Insight into Mechanism of Evolution of Mammalian Synthetic Prions

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Previous studies established that transmissible prion diseases could be induced by *in vitro*-produced recombinant prion protein (PrP) fibrils with structures that are fundamentally different from that of authentic PrP scrapie isoform (PrP^{Sc}). To explain evolution of synthetic prions, a new mechanism referred to as deformed templating was introduced. Here, we asked whether an increase in expression level of the cellular form of PrP (PrP^{C}) speeds up the evolution of synthetic strains *in vivo*. We found that in transgenic mice that overexpress hamster PrP^{C}, PrP^{C} overexpression accelerated recombinant PrP fibril-induced conversion of PrP^{C} to the abnormal proteinase K-resistant state, referred to as atypical PrPres, which was the first product of PrP^{C} misfolding *in vivo*. However, overexpression of PrP^{C} did not facilitate the second step of synthetic strain evolution-transition from atypical PrPres to PrP^{Sc}, which is attributed to the stochastic nature of rare deformed templating events. In addition, the potential of atypical PrPres to interfere with replication of a short-incubation time prion strain was investigated. Atypical PrPres was found to interfere strongly with replication of 263K *in vitro*; however, it did not delay prion disease in animals. The rate of deformed templating does not depend on the concentration of substrate and is hence more likely to be controlled by the intrinsic rate of conformational errors in templating alternative self-propagating states. *(Am J Pathol 2016, 186: 1006–1014; http://dx.doi.org/10.1016/j.ajpath.2015.11.013)*

Prion diseases, or transmissible spongiform encephalopathies (TSEs), are fatal neurodegenerative disorders that can arise spontaneously, be inherited, or be acquired through transmission.[1] The diversity in the cause of prion diseases can be explained by three mechanisms. Spontaneous TSE arises from the misfolding and aggregation of the normal, cellular isoform of the prion protein, PrP^{C}, into the disease-associated infectious scrapie isoform, PrP^{Sc}, and thus, underlies the sporadic forms of prion diseases.[2] Second, single point mutations or truncations in the *PRNP* gene cause inherited prion diseases via facilitating misfolding of PrP^{C}.[3–5] Third, in prion diseases acquired through the transmission, PrP^{Sc} seeds initiate prion replication by recruiting host PrP^{C}. According to the conventional view of template-assisted replication mechanisms, prions replicate with high fidelity; ie, the folding pattern of a newly formed PrP^{Sc} accurately reproduces that of the PrP^{Sc} template.[2]

Recent studies introduced a new mechanism, referred to as deformed templating, according to which transmissible prion disease can be induced by PrP amyloid fibrils with structures that are fundamentally different from that of PrP^{Sc}.[6–9] The mechanism of deformed templating was used to explain transformation of self-replicating states during evolution of prion strains of synthetic origin.[8,10–12] Amyloid fibrils produced from recombinant Syrian hamster PrP (rPrP) *in vitro* lacked any detectable PrP^{Sc} particles, yet they were able to trigger transmissible TSEs in animals on serial passaging.[8,10] In another illustration of deformed templating, noninfectious fibrils with a stacked β-sheet architecture, prepared from recombinant fungal prion protein HET-s, nucleated the infectious state of HET protein with a β-solenoid structure.[13] Further illustrations of the deformed templating mechanism are offered by the so-called quaking or amyloid seeding assays, in which infectious PrP^{Sc} nucleates noninfectious self-replicating fibrillar states when exposed to

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rPrP *in vitro*.[14,15] In direct support of the deformed templating mechanism, molecular imaging revealed that switching between alternative folding patterns can occur within individual amyloid fibrils.[16]

Previous studies established a mechanistic model for genesis of authentic PrP^{Sc} from noninfectious fibrils that involves two main steps (Figure 1).[8,10] In the first step, rPrP fibrils trigger PrP^{C} misfolding, a process that results in accumulation of an alternative self-replicating state, referred to as atypical PrPres. rPrP and atypical PrPres appear to be similar in structure, because both have a short, C-terminal proteinase K (PK)-resistant core.[8,10,17] In the second step, formation of authentic PrP^{Sc} is triggered via a relatively rare deformed templating event. After the first PrP^{Sc} particles are produced through deformed templating, PrP^{Sc} can replicate independently of atypical PrPres (Figure 1). This mechanism proposes that two barriers on a pathway from rPrP fibrils to PrP^{Sc} exist. The first barrier is attributed to a change in substrate from rPrP to PrP^{C}. In contrast to rPrP, PrP^{C} has a glycophosphatidylinositol-anchor and N-linked glycans, two types of post-translational modifications that might constrain conformational space acquired by self-replicating states.[18] The second barrier is attributable to a low rate of deformed templating, the events by which structures different from that of atypical PrPres are generated (Figure 1).

In the current study we tested whether substrate plays a role in evolution of authentic PrP^{Sc}. Would evolution of infectious PrP^{Sc} structures be faster or more efficient in animals overexpressing PrP^{C}? We found that, in transgenic (tg7) mice that overexpress hamster PrP^{C} on an ablated background,[19] high concentration of a substrate helped to overcome the first barrier, which is the transition from rPrP to PrP^{C} as a substrate and formation of atypical PrPres. However, overexpression of PrP^{C} did not facilitate crossing the second barrier, which is the transition from atypical PrPres to PrP^{Sc}. This result suggests that the rate of deformed templating is not controlled by PrP^{C} expression levels. Moreover, although PrP^{C} overexpression did not speed up the overall rate of evolution of synthetic prions, it expanded the range of self-replicating fibrillar states generated *in vitro* that were capable of inducing TSEs in animals.

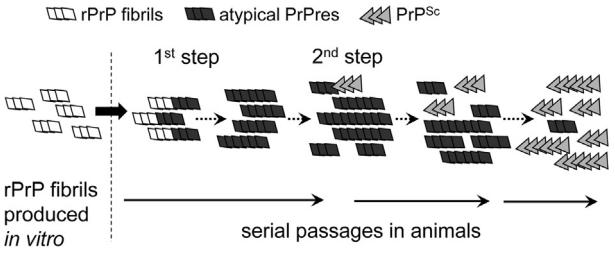

Figure 1 Schematic presentation of the mechanism, illustrating genesis of PrP^{Sc} triggered by rPrP fibrils. In a first step, rPrP fibrils seeded atypical PrPres, a transmissible form of PrP that replicates silently without causing clinical disease. Replication of atypical PrPres occasionally produces PrP^{Sc} in seeding events that appears to be rare and stochastic as described for a deformed templating mechanism. PrP^{Sc} replicates faster than atypical PrPres and eventually replaces it during serial passages. The two forms atypical PrPres and PrP^{Sc} can be distinguished after PK treatment via staining Western blot analyses with 3F4 and SAF-84 antibody. Because PK-resistant regions of atypical PrPres is shorter than that of PrP^{Sc}, it binds only SAF-84 antibody (epitope 160 to 170), whereas PrP^{Sc} binds both 3F4 (epitope 109 to 112) and SAF-84 antibodies. PK, proteinase K; atypical PrPres, alternative self-replicating state of prion protein; PrP^{Sc}, prion protein scrapie isoform; rPrP, recombinant prion protein.

Materials and Methods

Expression and Purification of rPrP and Formation of rPrP Fibrils

Syrian hamster full-length recombinant PrP that encompass residues 23 to 231 was expressed and purified as previously described,[20] with minor modifications.[9] Lyophilized rPrP was dissolved in 5 mmol/L HEPES, pH 7.0, immediately before use. To form fibrils for inoculations, a mixture of 0.5 mg/mL rPrP with 50 mmol/L 2-(*N*-morpholino)ethanesulfonic acid buffer, pH 6.0, and 2.0 mol/L guanidine hydrochloride (GdnHCl) was incubated at 37°C under continuous agitation. Amyloid formation was confirmed by thioflavin T fluorescence assay, epifluorescent microscopy, and electron microscopy as described previously.[20] For tg7 mouse inoculations, fibrils were diluted to 0.23 mg/mL rPrP and dialyzed into phosphate-buffered saline (PBS), pH 7.4. To prepare α-rPrP for inoculating the control group, rPrP stock solution was diluted in PBS to a final protein concentration of 0.23 mg/mL. Conformation of α-rPrP was confirmed by circular dichroism. Syrian hamsters were inoculated with fibrils at the concentration of 0.5 mg/mL rPrP dialyzed into PBS, pH 7.4.

Bioassay

All inoculations were performed intracerebrally, under 2% O_2/4 minimum alveolar concentration isoflurane anesthesia. Each mouse or hamster received 30 or 50 μL of inoculum, respectively, into the left hemisphere, approximately 3 mm to the left of the midline and approximately 3 mm anterior to a line drawn between the ears. After inoculation, animals were observed daily for disease with the use of a blind scoring protocol. For the first passage, weanling tg7 mice or Syrian hamsters were inoculated with rPrP amyloid fibrils or α-rPrP prepared as described above. Mice and hamsters were euthanized at 510 to 524 and 659 to 664 days after inoculation, respectively, without any sign of clinical disease, and their brains were removed aseptically and saved for analysis and second passage. For the second and third passages, 10% brain homogenates (BHs) prepared by homogenization in PBS, pH 7.4,[11] were dispersed by 30 seconds of sonication immediately before inoculation. For the experiment on interference, protein misfolding cyclic amplification with partially deglycosylated substrate (dgPMCA)-derived atypical PrPres produced as described below was diluted 10-fold with PBS supplemented with 1%