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EXTRA VIEWS

Structural conservation of prion strain specificities in recombinant prion protein fibrils in real-time quaking-induced conversion

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ABSTRACT. A major unsolved issue of prion biology is the existence of multiple strains with distinct phenotypes and this strain phenomenon is postulated to be associated with the conformational diversity of the abnormal prion protein (PrP^{Sc}). Real-time quaking-induced conversion (RT-QUIC) assay that uses *Escherichia coli*-derived recombinant prion protein (rPrP) for the sensitive detection of PrP^{Sc} results in the formation of rPrP-fibrils seeded with various strains. We demonstrated that there are differences in the secondary structures, especially in the β-sheets, and conformational stability between 2 rPrP-fibrils seeded with either Chandler or 22L strains in the first round of RT-QUIC. In particular, the differences in conformational properties of these 2 rPrP-fibrils were common to those of the original PrP^{Sc}. However, the strain specificities of rPrP-fibrils seen in the first round were lost in subsequent rounds. Instead, our findings suggest that nonspecific fibrils became the major species, probable owing to their selective growth advantage in the RT-QUIC. This study shows that at least some strain-specific conformational properties of the original PrP^{Sc} can be transmitted to rPrP-fibrils *in vitro*, but further conservation appears to require unknown cofactors or environmental conditions or both.

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IN VITRO *CONVERSION OF RECOMBINANT PrP INTO THE PROTEINASE K (PK) RESISTANT AMYLOID FIBRILS*

Prion diseases, or transmissible spongiform encephalopathies, are infectious and fatal neurodegenerative disorders that include Creutzfeldt-Jakob disease in humans, and scrapie and bovine spongiform encephalopathy in animals. The infectious agent, prion, is assumed to be formed mainly or exclusively by abnormal prion protein, designated PrP^{Sc}, which is partially protease-resistant[1] and a β-sheet-rich conformer,[2,3] frequently resulting in amyloid fibril formation. Although the pathogenesis has not been clarified fully, it is widely accepted that prion disease occurs through autocatalytic conformational conversion of the ubiquitous normal form of prion protein (PrP^{C}) to PrP^{Sc} in a "protein only" manner.[4]

Studies using *Escherichia coli*-derived purified recombinant PrP (rPrP) has contributed to solving the controversial protein-only hypothesis. It has been demonstrated that rPrP fibrils (rPrP-fibrils) formed *in vitro* cause the accumulation of PrP^{Sc} in the brains of PrP-overexpressing transgenic (Tg) mice[5–7] and some wild-type hamsters.[8] These studies suggest that rPrP can be converted into a PrP^{Sc}-like form *in vitro*; however, the infectious titers seem to be much lower than that of authentic PrP^{Sc}. In contrast, prion infectivity could be propagated when brain-derived PrP^{C} or baculovirus-derived PrP^{C} was used as substrates for protein misfolding cyclic amplification (PMCA) in the presence of certain cofactors such as nucleic acids.[9,10] Relatively high levels of prion infectivity was demonstrated by injection of PK-resistant rPrP-fibrils generated by unseeded PMCA in the presence of 1-palmitoyl-2-oleoylphosphatidylglycerol and total liver RNA into wild-type mice. Subsequently, these mice developed prion disease with an incubation period of approximately 150 days.[11] However, other group failed to show infectivity of rPrP-fibrils generated by the same methods.[12]

TRANSMISSION OF CONFORMATIONAL PROPERTIES OF PRION STRAINS TO rPrP-FIBRILS IN RT-QUIC

Prion is known to provide extensive strain diversity showing different phenotypic and pathological states in mammalian species. The strain-specific characteristics can usually be serially passaged stably in the same species. Furthermore, PrP^{Sc} generated by PMCA using brain homogenate from normal animals as a source of PrP^{C} (BH-PMCA) seeded with different mouse prion strains retained the strain-specific properties, such as incubation time, neuropathology, and biochemical characteristics from original PrP^{Sc}.[13] This result indicates that the intracellular mechanisms and cell-to-cell transmission are dispensable for the maintenance and propagation of strain characteristics. The finding that PrP^{Sc} from different strains have distinct secondary structures and biochemical properties supports the notion that prion strains are manifested by conformational variations of the PrP^{Sc}.[14] For example, strain-dependent differences in β-sheet-rich structures of PrP^{Sc} have been demonstrated by infrared spectroscopy.[15–18] In addition, the conformational stability of PrP^{Sc} differed among prion strains, as demonstrated by guanidine hydrochloride denaturation assay followed by protease digestion.[19,20] However, the mechanistic relationship between PrP^{Sc} conformational differences and the molecular basis of prion strains remains poorly understood.

The recently developed "real-time quaking-induced conversion" (RT-QUIC) is a sensitive

prion detection method, in which intermittent shaking enhances the conversion of soluble rPrP into amyloid fibrils in the presence of PrP^{Sc}.[21] Recent studies show that RT-QUIC assays allow highly sensitive detection of PrP^{Sc} in most species and strains, including Creutzfeldt-Jakob disease in humans,[21–24] scrapie in rodents,[25,26] and chronic wasting disease in cervids.[27]

We generated the amyloid fibrils seeded with 100 pg of PrP^{Sc} derived from either the Chandler or 22L strain in the first round of RT-QUIC (1^{st}-rPrP-fib^{Sc}).[28] Spontaneous formation of rPrP-fibrils (rPrP-fib^{spon}) was observed by decreasing the concentration of rPrP, because there was an inverse correlation between the rate of fibril formation and the concentration of rPrP. Previous studies using FTIR and hydrogen/deuterium exchange have shown that there are structural differences between PrP^{Sc}-seeded and spontaneous rPrP-fibrils generated by PMCA.[29,30] We found that the PK-**resistant** band pattern, structural morphology, secondary structure, and conformational stability distinguish 1^{st}-rPrP-fib^{Sc} from rPrP-fib^{spon}. Although there were no differences in the PK-**resistant** band pattern and structural morphology between Chandler-seeded (1^{st}-rPrP-fib^{Ch}) and 22L-seeded rPrP-fibrils (1^{st}-rPrP-fib^{22L}), we observed significant differences in the secondary structure and conformational stability between strains. FTIR analysis showed that native rPrP had an abundance of α-helical structures, whereas 1^{st}-rPrP-fib^{Ch} and 1^{st}-rPrP-fib^{22L} were substantially enriched in β-sheets. While the 1^{st}-rPrP-fib^{Ch} was characterized by a major band at 1624 cm^{-1} in the β-sheet region of second-derivative spectra, the 1^{st}-rPrP-fib^{22L} was characterized by 2 absorbance bands at 1629 and 1617 cm^{-1}, indicating that there were conformational differences in β-sheet structures between the 2 1^{st}-rPrP-fib^{Sc}. Similarly, purified Chandler-PrP^{Sc} from brains of mice displayed the spectrum with a peak at 1630 cm^{-1}, whereas purified 22L-PrP^{Sc} had 2 major maxima at 1631 and 1616 cm^{-1}, as previously reported. Thus, the differences in β-sheet spectrum shape between strains were common to both PrP^{Sc} and 1^{st}-rPrP-fib^{Sc}. The conformational stability of 1^{st}-rPrP-fib^{22L} was significantly lower than that of 1^{st}-rPrP-fib^{Ch}, as with Chandler- and 22L-PrP^{Sc}. Furthermore, wild-type mice inoculated with the 1^{st}-rPrP-fib^{Sc} showed an increased attack rate and a significantly shorter survival period compared with those inoculated with mock preparations. The infectious titers (per 40 μl) of 1^{st}-rPrP-fib^{Ch} and 1^{st}-rPrP-fib^{22L} were estimated to be 407.2 $\pm$ 226.6 and 1067.0 $\pm$ 678.7 LD_{50}, respectively, whereas the titers of Chandler and 22L prion were 20.2 and 28.9 LD_{50} units/40 pg of PrP^{Sc}, respectively, indicating that QUIC reaction in the first round resulted in a 20- to 37-fold increase in the infectious titer. These results suggest that strain features of PrP^{Sc} can be transmitted to rPrP-fibrils in a simple system solely consisting of pure rPrP. However, it is clear that the conformation of 1^{st}-rPrP-fib^{Sc} is not identical to that of authentic PrP^{Sc}. It should be noted that the degrees of vacuolation of mice inoculated with 1^{st}-rPrP-fib^{Sc} were significantly lower in the hippocampus and cerebellum than those of inoculated with mock preparations. The different lesion profiles may result from the conformational differences between 1^{st}-rPrP-fib^{Sc} and authentic PrP^{Sc}.

WHAT IS REQUIRED FOR MAINTAINING STRAIN-SPECIFIC CONFORMATIONS?

We found that the strain-specific conformational features and the infectivity disappeared in rPrP-fibrils during and after the second round,[28] suggesting that RT-QUIC has the limitation of technology with respect of reproducing the prion propagation. One possible reason for the loss of the prion strain-specific traits is that *E. coli*-derived rPrP lacks post-translational modifications. PrP bears 2 N-linked glycosylation sites at amino acids 180 and 196 that can produce di-, mono-, and unglycosylated forms. PrP^{Sc} has varying degrees of glycosylation among strains[14,31,32] and therefore the glycosylation pattern is postulated to confer strain specificity. Studies using Tg mice expressing glycosylation site mutants revealed that the strain properties of strain 79A were altered by the glycosylation state of PrP^{C}, but the strain

properties of strains ME7 and 301C were not affected.[33] Moreover, the glycosylation-deficient PrP^C as a substrate of PMCA by treatment with PNGase F did not affect strain-dependent neurotropisms in the 2 murine strains RML and 301C.[34] Furthermore, the cell tropisms determined by the cell panel assay were altered in strains RML, 139A, 79A, and ME7 but not in strain 22L when the strains were propagated in Tg mice expressing PrP devoid of a GPI anchor.[35] These results suggest that the necessity of glycans and the GPI-anchor for the transmission and preservation of strain-specific properties is dependent on the strains.

An additional reason for the loss of strain specificity from rPrP-fibrils might be because of a decrease in cofactor(s) over serial passages. Strains have been reported to differ in their RNA requirements for propagation in BH-PMCA, although RNA is not essential for maintaining strain-specific characteristics in mice.[36] Moreover, another study showed that phosphatidylethanolamine is a cofactor required for the propagation of prion infectivity in seeded rPrP-PMCA but not for the transmission of strain-specific properties, because 3 different prion strains changed into a single new strain after the serial passages of rPrP-PMCA reactions.[37,38] Thus, crucial cofactors or environmental conditions for maintaining strain-specific properties remain to be determined.

We observed the "nonspecific rPrP-fibrils" displayed no strain-specific differences in IR spectra and conformational stability after 5 serial rounds of RT-QUIC, which have the ability to cause the conversion of rPrP but failed to induce clinical signs of prion disease in the wild-type mice.[28] Additionally, we found that the β-sheet spectra of rPrP-fibrils generated in the presence of small amount (1 pg) of PrP^{Sc} or generated at pH4 in the first round were similar to nonspecific rPrP-fibrils.[28] These observations raise the possibility that nonspecific rPrP-fibrils lacking prion infectivity can be generated even in the first round and may interrupt the formation of the fibrils with strain-specific conforma-

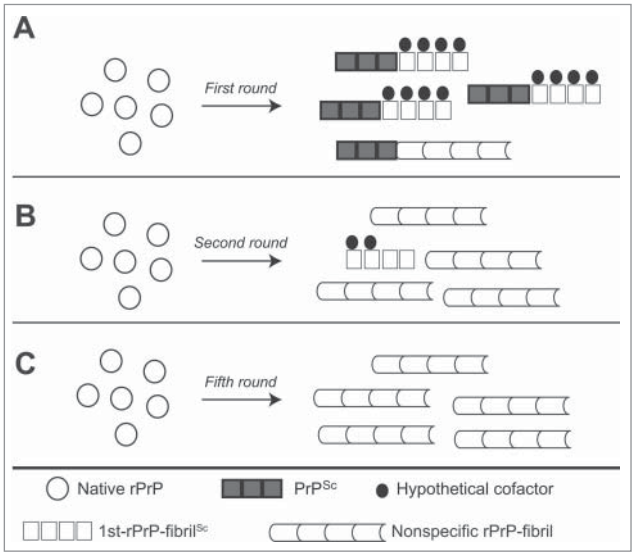

FIGURE 1. Hypothetical models for the formation of rPrP-fibrils in sequential RT-QUIC reactions. (**A**) The formation of 1^{st}-rPrP-fib^{Sc} is induced predominantly in the presence of hypothetical cofactors and brain-derived PrP^{Sc} in the first round. However, a small amount of nonspecific rPrP-fibrils may be concomitantly generated. (**B**) In the second round, the nonspecific rPrP-fibrils become predominant because of the paucity of hypothetical cofactors and/or a selective growth advantage of nonspecific fibrils. (**C**) The formation of nonspecific rPrP-fibrils occupies almost the whole reaction in the fifth round.

tions, because of a selective growth advantage of nonspecific fibrils (**Fig. 1**). Of note, the formation of quasi-species that is consisting of a variety of conformational variants has been reported in prion-infected cultured cells under different environmental conditions.[39,40] Furthermore, different prion strains can interfere with each other, and this is known as prion strain interference.[41–44] The competition for substrates among the variants is thought to act as a selection pressure in Darwinian evolution and to cause the phenomenon of prion strain interference. Previous work showed that 2 conformational variants of rPrP-fibrils are mutually exclusive and compete for monomeric rPrP as a substrate in the rPrP-PMCA.[29] Likewise, competitive amplification of 2 prion strains was observed in BH-PMCA.[45] We postulate that PrP^{Sc} predominantly leads to strain-specific conformational conversion of rPrP, particularly in the presence of hypothetical cofactors, while some quantity of nonspecific fibrils could be generated simultaneously in the first round (**Fig. 1A**). The conditions of subsequent rounds would favor growth of nonspecific species (**Fig. 1B and C**). The fact that prion infectivity was often diminished in serial rPrP-PMCA[46] or BH-PMCA[47–49] support the hypothesis that the amplification of nonspecific rPrP fibrils is accelerated by certain conditions. Further studies are needed to ascertain the key factors responsible for maintaining the infectious and strain-specific conformations *in vitro*.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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