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Conformational properties of prion strains can be transmitted to recombinant prion protein fibrils in real-time quaking-induced conversion

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Abstract

The phenomenon of prion strains with distinct biological characteristics has been hypothesized to be involved in the structural diversity of abnormal prion protein (PrPSc). However, the molecular basis of the transmission of strain properties remains poorly understood. Real-time quaking-induced conversion (RT-QUIC) is a cell-free system that uses *E. coli*-derived recombinant PrP (rPrP) for the sensitive detection of PrPSc. To investigate whether properties of various prion strains can be transmitted to amyloid fibrils consisting of rPrP (rPrP-fibrils) using RT-QUIC, we examined the secondary structure, conformational stability and infectivity of rPrP-fibrils seeded with PrPSc derived from either the Chandler or 22L strain. In the first round of the reaction there were differences in the secondary structures, especially in bands attributed to β-sheets, as determined by infrared spectroscopy, and conformational stability between Chandler-seeded (1st-rPrP-fibCh) and 22L-seeded rPrP-fibrils (1st-rPrP-fib22L). Of note, specific identifying characteristics of the two rPrP-fibril-types seen in the β-sheets resembled those of the original PrPSc. Furthermore, the conformational stability of 1st-rPrP-fibCh was significantly higher than that of 1st-rPrP-fib22L, as with Chandler- and
22L-PrP\textsuperscript{Sc}. The survival periods in mice inoculated with 1\textsuperscript{st}-rPrP-fib\textsuperscript{Ch} or 1\textsuperscript{st}-rPrP-fib\textsuperscript{22L} were significantly shorter than those of the mice inoculated with mock 1\textsuperscript{st}-QUIC mixtures. In contrast, these biochemical characteristics were no longer evident in subsequent rounds, suggesting that nonspecific uninfected rPrP-fibrils became predominant probably because of their rapid growth rate. Together, these findings show that at least some strain-specific conformational properties can be transmitted to rPrP-fibrils and unknown cofactors or environmental conditions may be required for further conservation.

**Importance**

The phenomenon of prion strains with distinct biological characteristics is assumed to result from the conformational variations in the abnormal prion protein (PrP\textsuperscript{Sc}). However, important questions remain about the mechanistic relationship between the conformational differences and the strain diversity, including how to transmit strain-specific conformations. In this study, we investigated whether properties of diverse prion strains can be transmitted to amyloid fibrils consisting of *E. coli*-derived recombinant PrP (rPrP) generated in the real-time quaking-induced conversion
(RT-QUIC), a recently-developed *in vitro* PrP\textsuperscript{Sc} formation method. We demonstrate that at least some of the strain-specific conformational properties can be transmitted to rPrP-fibrils in the first round of RT-QUIC by examining the secondary structure, conformational stability and infectivity of rPrP-fibrils seeded with PrP\textsuperscript{Sc} derived from either the Chandler or 22L prion strain. We believe that these findings will advance our understanding of the conformational basis underlying prion strain diversity.

**Introduction**

Prion diseases, or transmissible spongiform encephalopathies (TSE), are infectious and fatal neurodegenerative disorders characterized by progressive spongiform changes and the accumulation of abnormal prion protein (PrP\textsuperscript{Sc}) in the central nervous system. Although the pathogenic mechanisms have not been fully elucidated, prion disease is thought to occur through autocatalytic conversion of normal prion protein (PrP\textsuperscript{C}) to PrP\textsuperscript{Sc} (1, 2), known as the protein-only hypothesis. Some biophysical properties are known to differ between PrP\textsuperscript{C} and PrP\textsuperscript{Sc}. PrP\textsuperscript{C} is monomeric, detergent-soluble and protease-sensitive, while PrP\textsuperscript{Sc} is polymeric, detergent-insoluble and partially
protease-resistant (3). These differences are most likely due to the different conformations of the two isoforms. PrPC is largely \( \alpha \)-helical, whereas PrP\( \text{Sc} \) is substantially enriched in \( \beta \)-sheets (4, 5), frequently resulting in amyloid fibril formation.

The existence of diverse prion strains in mammalian species manifesting in phenotypic differences is well known. The strain-specific characteristics are usually maintained upon serial passage in the same species, and may be explained by conformational variations in the PrP\( \text{Sc} \). Indeed, strain-dependent differences in \( \beta \)-sheet-rich structures of PrP\( \text{Sc} \) have been demonstrated by infrared spectroscopy (6-9).

In addition, the conformational stability of PrP\( \text{Sc} \) differed among prion strains, as demonstrated by guanidine hydrochloride (GdnHCl) denaturation assay followed by protease digestion (10, 11). However, the mechanistic relationship between PrP\( \text{Sc} \) conformational differences and the molecular basis of prion strains remains poorly understood.

Various \textit{in vitro} PrP\( \text{Sc} \) formation methods have been developed to elucidate the pathogenesis of the prion diseases. One of these methods, protein misfolding cyclic amplification (PMCA), enabled an exponential amplification of PrP\( \text{Sc} \) \textit{in vitro} by
sonication-induced fragmentation of large PrP\textsuperscript{Sc} polymers into smaller units (12). The amplified PrP\textsuperscript{Sc} was accompanied by an increase in infectivity using normal brain homogenate (BH) as a source of PrP\textsuperscript{C} substrates (BH-PMCA) (13). Furthermore, PrP\textsuperscript{Sc} generated by BH-PMCA from five different mouse prion strains retained the strain-specific properties (14). In addition, prion infectivity could be propagated when purified brain-derived PrP\textsuperscript{C} or baculovirus-derived PrP\textsuperscript{C} was used as substrates in the presence of certain cofactors such as nucleic acids and BH from PrP-deficient mice (15-17). These results provide strong evidence to support the protein-only hypothesis, but the structural basis of prion pathogenesis, including the tertiary structure of PrP\textsuperscript{Sc}, has not been fully clarified.

On the other hand, the use of \textit{E. coli}-derived purified recombinant PrP (rPrP) offers an advantage over conformational analyses, which generally require a high purity and a large quantity of the target protein. Spontaneously-polymerized amyloid fibrils of rPrP have been reported to induce the accumulation of PrP\textsuperscript{Sc} in the brains of PrP-overexpressing transgenic (Tg) mice (18-20) and some wild-type hamsters (21), however the incubation periods spanned no less than several hundred days and none of
the wild-type hamsters developed any neurological signs at first passage, indicating that
the level of infectivity generated in these studies is very low. More recently, wild-type
mice developed clinical disease typical of TSE around 130 days after injection of
proteinase K-resistant rPrP fibrils (rPrP-fibrils) generated by unseeded-PMCA in the
presence of 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG), a synthetic lipid
molecule, and total liver RNA (22). Although these results were reproduced by the same
group (23), others have reported that rPrP-fibrils generated by the same method were
unable to induce either neuropathological changes or the accumulation of PrP\textsuperscript{Sc} (24).
Thus, the role of POPG and RNA in the de novo generation of infectious rPrP-fibrils
remains controversial.

Meanwhile, two different seeded-PMCA reaction studies using rPrP
(rPrP-PMCA) as a substrate have demonstrated the propagation of moderate levels of
prion infectivity. One study showed that hamster rPrP can be converted to rPrP-fibrils
capable of inducing TSE in the presence of SDS, a synthetic anionic detergent, but there
were great variations in the attack rate and the incubation period, which ranged from 119
to 401 days (25). Another study revealed that phosphatidylethanolamine (PE), a
phospholipid found in biological membranes, enhances conversion of mouse rPrP into rPrP-fibrils capable of inducing TSE after around 400 days of incubation periods with a 100% attack rate (26, 27). Of note, three different strains used as a seed were converted into a single strain with unique strain properties during the serial rPrP-PMCA experiments (27). These studies suggest that a certain amphipathic molecule such as PE is a required cofactor for the propagation of prion infectivity in vitro, but not for the transmission of strain-specific properties.

The recently developed “real-time quaking-induced conversion” (RT-QUIC) is a sensitive prion detection method (28, 29), in which intermittent shaking enhances the conversion of soluble rPrP into amyloid fibrils in the presence of PrPSc. The aim of the present research was to investigate whether properties of diverse prion strains can be transmitted to rPrP-fibrils generated in the RT-QUIC. We produced proteinase K-resistant rPrP-fibrils seeded with minute quantities of mouse-adapted scrapie (Chandler or 22L strain) PrPSc and investigated the secondary structure, conformational stability and infectivity.
MATERIALS AND METHODS

Recombinant mouse PrP expression and purification

Recombinant PrP (rPrP) equivalent to residues 23-231 of the mouse PrP sequence was expressed, refolded into a soluble form, and purified essentially as previously described (30). The concentration of rPrP was determined by measuring the absorbance at 280 nm.

The purity of the final protein preparations was ≥ 99 %, as estimated by SDS-PAGE, immunoblotting and liquid chromatography-mass spectrometry (data not shown). After purification, aliquots of the proteins were stored at -80 °C in 10 mM phosphate buffer, pH 6.8 or distilled water.

Preparation of brain homogenates

Brain tissues were homogenized at 10% (w/v) in ice-cold PBS supplemented with a protease inhibitor mixture (Roche) using a multi-bead shocker (Yasui Kikai, Osaka, Japan). After centrifugation at 2,000 g for 2 min, supernatants were collected and frozen at -80 °C until use. Total protein concentrations were determined by the BCA protein
The PrP<sub>Sc</sub> concentrations in the brain homogenates were estimated by dot-blot analysis using a reference standard of rPrP, as previously described (31).

**RT-QUIC experiments**

We prepared reaction mixtures in a 96-well, optical, black bottom plate (Nunc 265301) to a final total volume of 100 µl. To avoid contamination, we prepared non-infectious materials inside a biological safety cabinet in a prion-free laboratory and used aerosol-resistant tips. The final concentrations of reaction buffer components were 300 mM NaCl, 50 mM HEPES pH 7.5, and 10 µM Thioflavin T (ThT). The concentration of rPrP was 50 or 100 µg/ml, and only freshly-thawed rPrP was used. Brain homogenate was diluted with reaction buffer prior to the reactions. The 96-well plate was covered with sealing tape (Nunc 236366) and incubated at 40 °C in a plate reader (Infinite M200 fluorescence plate reader, TECAN) with intermittent shaking, consisting of 30 s of circular shaking at the highest speed and no shaking for 30 s, with a 2 min pause to measure the fluorescence. The kinetics of amyloid formation was monitored by the bottom reading of the fluorescence intensity every 10 min using 440-nm excitation and
485-nm emission wavelength of monochromators.

**RT-QUIC products analysis**

For detection of protease-resistant rPrP, 10 μl of the QUIC samples (1 μg of rPrP) was diluted with 40μl of buffer (300 mM NaCl, 50 mM HEPES pH 7.5) and digested with 10 μg/ml of proteinase K (PK) at 37 °C for 1 h. After adding Pefabloc (Roche) at a final concentration of 4 mM and 20 μg of thyroglobulin, the proteins were precipitated with 4 volumes of methanol. The samples were heated in sample buffer (2% SDS, 5% β-mercaptoethanol, 5% sucrose, 0.005% bromophenol blue and 62.5 mM Tris-HCl; pH 6.8) at 95 °C for 5 min, and then loaded onto 10% BisTris NuPAGE gels (Invitrogen). Proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were probed with polyclonal anti-PrP antibody R20 (epitope located at mouse PrP amino acids 218-231) or ICSM35 (D-Gen, London, UK).

**Transmission electron microscopy**

Negative staining was done on carbon supporting film grids, which were glow-discharged
before staining. The 10 µl samples were adsorbed to the grids for 3 min, then the residual solution was absorbed by filter paper. The grids were stained with 20 µl of fleshly filtered stain (2% uranyl acetate). Once dry, the samples were viewed in a transmission electron microscope (JEM-1200EX, JEOL, Japan).

Fourier transform infrared spectroscopy (FTIR)

FTIR spectra were measured with a Bruker Tensor 27 FTIR instrument (Bruker Optics) equipped with an MCT detector cooled with liquid nitrogen. 300 µl each of the QUIC samples (30 µg of rPrP) were pelleted by centrifugation for 1 h at 77,000 g, and resuspended in 20 µl buffer (300 mM NaCl, 50 mM HEPES pH 7.5). The slurry was loaded into BioATRcell II. PrP<sup>Sc</sup> was purified from the brains of mice infected with the mouse-adapted Chandler and 22L prion using a combination of detergent solubilization, centrifugation at ultrahigh speeds and PK digestion (4, 32), and 15 µl of purified PrP<sup>Sc</sup> were directly loaded. 128 scans at 4 cm<sup>−1</sup> resolution were collected for each sample under constant purging with nitrogen, corrected for water vapor, and background spectra of buffer were subtracted.
Conformational stability assay

10 µl of the QUIC products (equivalent to 1 µg of rPrP) and brain homogenates (80 µg of total proteins) were mixed with 22 µl of various concentrations of guanidine hydrochloride (GdnHCl) at a final concentration of 0 to 5 M and 0 to 3.5 M, respectively, and the mixed samples were incubated at 37 °C for 1h. After adjusting the final GdnHCl concentration of the QUIC products to 1 M and the brain homogenates to 0.6 M, the samples were digested with PK (10 µg/ml) at 37 °C for 1h, and analyzed by Western blotting following methanol precipitation. The bands were visualized using Attophos AP Fluorescent Substrate system (Promega) and quantified using Molecular Imager FX (BIO-RAD). The sigmoidal patterns of denaturation curves were plotted using a Boltzmann curve fit. The concentration of GdnHCl required to denature 50% of PK-resistant fragments ([GdnHCl]_{1/2}) was estimated from the denaturation curves.

Bioassay

Male 4-week-old ddY mice were intracerebrally inoculated with 40 µl of QUIC products
(equivalent to 4 µg rPrP). As controls for rPrP-fibrils, we performed a mock QUIC procedure using seed-only solutions that contained the same concentration of PrP<sup>Sc</sup> as 1<sup>st</sup>-rPrP-fibril (1 pg/µl) or 5<sup>th</sup>-rPrP-fibril (1 × 10<sup>-8</sup> pg/µl), then added the same amount of rPrP, and inoculated the mixtures into mice. Brain homogenates were serially diluted with PBS, from 10<sup>0</sup> to 10<sup>-7</sup>, and 20 µl of each dilution was intracerebrally inoculated. Mice were monitored weekly until the terminal stage of disease or sacrifice. Clinical onset was determined as the presence of 3 or more of the following signs: greasy and/or yellowish hair, hunchback, weight loss, yellow pubes, ataxic gait and nonparallel hind limbs. The 50% lethal dose (LD<sub>50</sub>) was determined according to the Behrens-Karber formula. Animals were cared for in accordance with the Guidelines for Animal Experimentation of Nagasaki University.

**Histopathology and Lesion Profiles**

The brain tissue was fixed in 4% paraformaldehyde, and 5 µm paraffin sections prepared on PLL coat slides using a microtome. After deparaffinization and rehydration, the tissue sections were stained with hematoxylin and eosin. The pattern of vacuolation was
examined in 8 fields per slice from the hippocampus, cerebral cortex, hypothalamus, pons and cerebellum. Spongiform degeneration was scored using the following scale: 0, no vacuoles; 1, a few vacuoles widely and unevenly distributed; 2, a few vacuoles evenly scattered; 3, moderate numbers of vacuoles evenly scattered; 4, many vacuoles with some confluences; 5, dense vacuolation.

**Statistical Analysis**

The fibril-length or width determined by electron microscopy analysis was subjected to one-way analysis of variance (ANOVA) followed by Tukey-Kramer test. The data of the conformational stability test was analyzed by one-way ANOVA followed by student’s t-test. The data analysis of the survival times was evaluated by Logrank test. The data of the vacuolation score was analyzed by Mann-Whitney’s U test.
Results

Conversion of the soluble form of mouse recombinant PrP into amyloid fibrils by RT-QUIC

We first tested whether formation of mouse rPrP amyloid fibrils could be induced in the RT-QUIC by monitoring levels of ThT fluorescence. We observed positive ThT fluorescence in the presence of diluted Chandler-brain homogenate (BH) or 22L-BH containing 100 pg of PrPSc (Fig. 1A), whereas negative control reactions seeded with comparable dilutions of normal brain homogenate (NBH) or without seed resulted in no increase in ThT fluorescence over 72 h (Fig. 1A). However, because an inverse correlation existed between the rate of fibril formation and the concentration of rPrP (28, 33), spontaneous formation of rPrP-fibrils (rPrP-fibspon) was induced by decreasing the concentration of rPrP from 100 to 50 μg/ml (Fig. 1A).

We next examined the PK-resistance of rPrP-fibrils by immunoblotting using anti-PrP antibody R20 directed toward C-terminal residues 218–231. Although the ThT-negative reactions seeded with NBH or without seed produced no PK-resistant bands (Fig. 1B middle panel), the Chandler-seeded rPrP-fibrils (rPrP-fibCh) and
22L-seeded rPrP-fibrils (rPrP-fib<sup>22L</sup>) produced several (21-, 18-, 12-, 11- and 10-kDa) PK-resistant fragments (Fig. 1B, left panel). In contrast, the PK digestion of rPrP-fib<sup>spon</sup> generated only 10-12 kDa fragments. It should be noted that anti-PrP monoclonal antibody ICSM35 (directed toward an epitope consisting of residues 93-102) specifically recognized the 21- and 18 kDa fragments derived from PrP<sup>Sc</sup>-seeded rPrP-fibrils in the first round (1<sup>st</sup>-rPrP-fib<sup>Sc</sup>), indicating that they contained mouse PrP from about residues 93-231 (Fig. 1B, right panel).

To further characterize the structure of 1<sup>st</sup>-rPrP-fib<sup>Sc</sup> and rPrP-fib<sup>spon</sup>, the samples were examined using a negative-stained transmission electron microscope (TEM). The electron micrographs of 1<sup>st</sup>-rPrP-fib<sup>Ch</sup> and 1<sup>st</sup>-rPrP-fib<sup>22L</sup> revealed bundles of irregularly rod-shaped and branched fibrils, while most rPrP-fib<sup>spon</sup> displayed smooth and non-branched rod-shaped fibrils (Fig. 1C). Moreover, the lengths of 1<sup>st</sup>-rPrP-fib<sup>Ch</sup> and 1<sup>st</sup>-rPrP-fib<sup>22L</sup> were significantly longer than that of rPrP-fib<sup>spon</sup> (Fig. 1D). Thus, the results of TEM analysis suggest that 1<sup>st</sup>-rPrP-fib<sup>Sc</sup> is structurally distinct from spontaneous rPrP-fib<sup>spon</sup>.

We next examined the morphology of PrP<sup>Sc</sup>-seeded rPrP-fibrils in the second
and fifth round reactions (2\textsuperscript{nd}- and 5\textsuperscript{th}-rPrP-fib\textsuperscript{Sc}) by TEM. In contrast to 1\textsuperscript{st}-rPrP-fib\textsuperscript{Sc}, 2\textsuperscript{nd}- and 5\textsuperscript{th}-rPrP-fib\textsuperscript{Sc} displayed spindly and non-branched fibrils or amorphous aggregates (Fig. 2). These data support the view that 1\textsuperscript{st}-rPrP-fib\textsuperscript{Sc} are structurally distinct from those of 2\textsuperscript{nd}- and 5\textsuperscript{th}-rPrP fib\textsuperscript{Sc}.

**Structural characterization of rPrP-fibrils by FTIR**

We next examined the secondary structure of rPrP-fibrils and purified PrP\textsuperscript{Sc} from brains of mice infected with Chandler or 22L scrapie by FTIR. A silver-stained SDS-PAGE gel analysis revealed that Chandler- and 22L-PrP\textsuperscript{Sc} preparations were highly purified (Fig. 3A). Furthermore, TEM analysis demonstrated that the PrP\textsuperscript{Sc} preparations consisted exclusively of amyloid-like fibrils (Fig. 3B). FTIR analysis showed that Chandler-PrP\textsuperscript{Sc} was characterized by a major band at 1630 cm\textsuperscript{-1} in the β-sheet region of second-derivative spectra, while 22L-PrP\textsuperscript{Sc} was characterized by two absorbance bands at 1631 and 1616 cm\textsuperscript{-1} (Fig. 4A), indicating that there were conformational differences in β-sheet structures between Chandler- and 22L-PrP\textsuperscript{Sc}, as previously reported (7). Consistent with previous reports (6-9), bands of around 1656–1658 cm\textsuperscript{-1} were observed in both Chandler- and
22L-PrPSc. Although these bands were formerly attributed to α-helix, recent studies using direct mass spectrometric analysis of hydrogen/deuterium exchange and FTIR analysis have suggested that purified PrPSc has little α-helix content, and the bands probably result from turns (9, 34). Native rPrP had maximum absorbance at 1653 cm⁻¹, which was congruent with that of prominent α-helical structures. In contrast, all rPrP-fibrils displayed prominent bands at lower wavenumbers (1630-1610 cm⁻¹), indicating predominantly β-sheet content (Fig. 4A). The β-sheet spectra revealed conformational differences among rPrP-fibSp, 1st-rPrP-fibCh and 1st-rPrP-fib22L. The rPrP-fibSp had a prominent band at 1623 cm⁻¹ and a modest band at 1610 cm⁻¹. While the 1st-rPrP-fibCh was characterized by a single major band at 1624 cm⁻¹, the 1st-rPrP-fib22L had two major maxima at 1629 and 1617 cm⁻¹ (Fig. 4A). Although 1st-rPrP-fibSc lacked the bands around 1656–1658 cm⁻¹, the strain-specific shapes (one peak in Chandler versus two peaks in 22L) in the β-sheet spectrum of the purified PrPSc resembled those of 1st-rPrP-fibSc.

To test whether the strain-specific IR spectra observed in 1st-rPrP-fibCh and 1st-rPrP-fib22L are transmitted to sequential QUIC reactions, we performed 5 serial rounds of QUIC (Supplementary Fig. 2). There was little difference in β-sheet spectra between
5th-rPrP-fib\textsuperscript{Ch} and 5th-rPrP-fib\textsuperscript{22L} (Fig 3), suggesting that strain-specific conformations were lost in the 5th-rPrP-fib\textsuperscript{Sc}. Furthermore, additional experiments revealed that infrared spectra of rPrP-fibrils produced in the presence of low amount of PrP\textsuperscript{Sc} (1 pg) or under acidic conditions (pH 4) displayed little differences between strains (Fig. 4B).

Conformational stability analysis of rPrP-fibrils and PrP\textsuperscript{Sc}

To examine the biochemical differences of rPrP-fibrils and PrP\textsuperscript{Sc} in BH between strains, we performed a conformational stability assay, which combines GdnHCl denaturation with PK digestion. The [GdnHCl]\textsuperscript{1/2} values for Chandler- and 22L-PrP\textsuperscript{Sc} were 3.3 ± 0.4 and 1.7 ± 0.3 M, respectively (Fig. 5A and Table 1), indicating that the conformational stability of Chandler-PrP\textsuperscript{Sc} was significantly higher than that of 22L-PrP\textsuperscript{Sc}. Consistent with previous work (11), Chandler-PrP\textsuperscript{Sc} bands treated with more than 1.5 M GdnHCl were approximately 5 kDa smaller than those treated with lower concentrations (Fig. 5A, upper panel). The [GdnHCl]\textsuperscript{1/2} of 1st-rPrP-fib\textsuperscript{Ch} and 1st-rPrP-fib\textsuperscript{22L} were 3.3 ± 0.1 and 2.3 ± 0.6 M, respectively (Fig. 5B and Table 1), showing that the stability of 1st-rPrP-fib\textsuperscript{Ch} was significantly higher than that of
1st-rPrP-fib^{22L}, as with Chandler- and 22L-PrP^{Sc}. Thus, the relationship between Chandler and 22L in terms of conformational stability was common to both the original PrP^{Sc} and 1st-rPrP-fib^{Sc}. In contrast, the [GdnHCl]_{1/2} of rPrP-fib^{^*^p^o^n} was more than 5 M, which was markedly higher than those of the 1st-rPrP-fib^{Sc} (Fig. 5B and Table 1). Additionally, we tested the conformational stability of 2nd- and 5th-rPrP-fib^{Sc}, but found no significant differences between strains (Fig. 5C, D and Table 1).

Bioassay for rPrP-fibrils generated in QUIC reactions

To determine whether the infectivity was transmitted to the rPrP-fibrils, we performed a bioassay using wild-type mice. To prepare the control materials, seed-only solutions containing the same concentration of PrP^{Sc} as 1st- or 5th-rPrP-fib^{Sc} were subjected to a mock RT-QUIC procedure and then mixed with the same amount of soluble rPrP (Table 2). The survival periods in mice inoculated with 40 µl aliquots containing rPrP-fibrils were 185.5 ± 4.0 days post-inoculation (dpi) for 1st-rPrP-fib^{Ch} and 213.0 ± 8.9 dpi for 1st-rPrP-fib^{22L} (Table 2). In contrast, the attack rate of these control mice was only 50% (2/4) for Chandler and 20% (1/5) for 22L. Moreover, the survival
periods of the affected mice were much longer than that of the mice inoculated with 1st-rPrP-fibSc (Table 2). For comparison with the 50% lethal dose (LD50) of the original PrPSc, the LD50 of 1st-rPrP-fibSc was determined by the linear regression relationship between infectious titers and survival periods. The infectious titers (per 40 μl) of 1st-rPrP-fibCh and 1st-rPrP-fib22L were estimated to be 407.2 ± 226.6 and 1067.0 ± 678.7 LD50, respectively, whereas the titers of Chandler and 22L prion were 20.2 and 28.9 LD50 units/40 pg of PrPSc, respectively. Because QUIC reaction in the first round resulted in a 20- to 37-fold increase in the infectious titer, a seed contribution to the infectivity is estimated to be around 3–5%. In contrast, none of the mice inoculated with 5th-rPrP-fibSc developed symptoms related to TSE (Table 2), suggesting that the 5th-rPrP-fibSc has no substantial infectivity.

We analyzed the levels of PrPSc in the brain tissues of terminal-stage mice inoculated with 1st-rPrP-fibSc or control materials (Mock 1st-QUIC) by Western blotting and found no apparent differences in the accumulation of PrPSc between them and the Mock 1st-QUIC (Fig. 6A). In addition, a conformational stability assay with GdnHCl revealed that the strain-specific digestion pattern was preserved in mice inoculated with
Next, the degree of vacuolation in brain sections including the hippocampus (HI), cerebral cortex (Cx), thalamus (TH), pons (Po) and cerebellum (CE) from affected mice inoculated with 1st-rPrP-fib<sup>Sc</sup> or Mock 1st-QUIC and those in the second passage of 1st-rPrP-fib<sup>Sc</sup> was examined histologically (Fig. 6C, D). Of note, we found that spongiform change of 1st-rPrP-fib<sup>Sc</sup>-inoculated mice was less severe in HI and CE than that of Mock 1st-QUIC (Fig. 6C, D). Furthermore, these different lesion profiles observed in 1st-rPrP-fib<sup>Sc</sup>-inoculated mice were preserved upon second passage (Fig. 6D), suggesting that 1st-rPrP-fib<sup>Sc</sup> are partially distinct from the original strains. These findings support the notion that 1st-rPrP-fib<sup>Sc</sup> provoke the emergence of a mutant strain beyond seed-derived infectivity.

**DISCUSSION**

Recent studies show that RT-QUIC assays are useful for the sensitive detection of PrP<sup>Sc</sup> in most species and strains, including Creutzfeldt-Jakob disease (CJD) in humans (28, 35-37), scrapie in rodents (29, 38), and chronic wasting disease (CWD) in cervids.
In the RT-QUIC reaction, soluble rPrP is converted to amyloid fibrils in a seed-dependent fashion in the presence of PrPSc. Previous studies using FTIR and hydrogen/deuterium exchange have shown that there are structural differences between PrPSc-seeded and spontaneous rPrP-fibrils generated in the rPrP-PMCA (7, 40). We also found that the structural morphology (Fig. 1C), secondary structure (Fig. 3) and conformational stability (Fig. 4B and Table 1) distinguish 1st-rPrP-fibSc from rPrP-fibspn. However, it has been unknown whether rPrP retains the conformational properties of the original PrPSc in the RT-QUIC. Consistent with previous reports (7, 11), we observed strain differences in β-sheet structure and conformational stability of PrPSc between Chandler and 22L strains. Likewise, the differences in β-sheet spectrum shape between strains were common to both PrPSc and 1st-rPrP-fibSc. Furthermore, the conformational stability of 1st-rPrP-fib22L was significantly lower than that of 1st-rPrP-fibCh, as with Chandler- and 22L-PrPSc. Since the original PrPSc remaining in the 1st-rPrP-fibSc was equivalent to only about 0.01–0.02% of PK-resistant 1st-rPrP-fibSc (1–2 µg/10 µg of total PrP) in our estimation, the contribution to the FTIR spectra and the conformational stability of 1st-rPrP-fibSc is considered to be negligible. Taken together, these studies
demonstrate that at least some strain-specific conformational features, especially in the β-sheet region, are conserved between PrPSc and 1st-rPrP-fibSc. However, these unique structural features disappeared in subsequent rounds.

One of the reasons for the loss of strain-specificity may be due to differences between E. coli-derived rPrP and brain-derived PrPc. Studies using circular dichroism and 1H-NMR spectroscopy showed that the tertiary structure and the thermal stability of bovine rPrP(23-230) are essentially identical to those of healthy calf brain-derived PrPc (41). However, it should be noted that E. coli-derived rPrP lacks posttranslational modifications of PrPc such as glycosylation and a glycosylphosphatidylinositol (GPI)-anchor. PrP has two N-linked glycosylation sites at amino acids 180 and 196, resulting in di-, mono- and unglycosylated forms. Mature PrPc is rich in the di-glycosylated form, whereas the glycoform ratio of PrPSc is known to vary among strains (42-44). Studies using PrP glycan-lacking Tg mice revealed that the strain-specific characteristics of 79A strain were affected in by the glycosylation status of PrPc, but ME7 and 301C strains were not (45). Meanwhile, enzymatic deglycosylation of PrPc failed to affect strain-specific pathological changes in serial PMCA experiments.
seeded with two murine strains, RML and 301C (46). However, the same two strains were converted into a new single strain during serial rPrP-PMCA in the presence of synthetic PE (27). Similarly, the emergence of mutant strains whose lesion profiles differ from that of the seed strain was also observed in the bioassay using hamster rPrP-fibrils generated in seeded rPrP-PMCA (25) or 1st-rPrP-fibSc (Fig. 6C, D). These results raise the possibility that the lack of a GPI-anchor in rPrP leads to alterations in the strain-specific characteristics. Furthermore, the cell tropisms determined by the Cell Panel Assay were altered in RML, 139A, 79A and ME7 strains but not in 22L when propagated in Tg mice expressing PrP devoid of a GPI-anchor (47). These studies demonstrate that glycosylation and a GPI-anchor are not necessarily required for the propagation of prion infectivity, but can influence the strain properties. Although the molecular basis of the emergence of mutant strains remains elusive, we can speculate that the posttranslational changes to PrP might affect the conformation of PrPSc or the interaction with some cofactor(s) in a strain-specific manner.

Another possible explanation is that nonspecific rPrP-fibrils are generated during the serial RT-QUIC, and replicate more rapidly than the fibrils with strain-specific
conformation. The term “nonspecific rPrP-fibrils” arises from our findings that there was little difference in IR spectra and conformational stability of 5\textsuperscript{th}-rPrP-fib\textsuperscript{Sc} between strains. It has been reported that the propagation of prion strains in cells cultured under different environments often leads to the formation of quasi-species that are assumed to be composed of a variety of conformational variants (48, 49). Once generated, the competition among the variants is thought to occur during propagation. Indeed, two conformational variants of rPrP-fibrils have been shown to be mutually exclusive and compete for monomeric rPrP as a substrate in the fibril formation (30). Furthermore, competitive amplification of two prion strains was demonstrated by BH-PMCA (50).

Similarly, nonspecific rPrP-fibrils would be expected to become the majority if they had a selective growth advantage in the RT-QUIC. We found that the β-sheet spectra of rPrP-fibrils generated in the presence of low amount (1 pg) of PrP\textsuperscript{Sc} or rPrP-fibrils generated at pH 4 in the first round were similar to those seen in 5\textsuperscript{th}-rPrP-fib\textsuperscript{Sc} (Fig. 4B). These observations also support this hypothesis and suggest that the amplification of nonspecific rPrP-fibrils is accelerated by certain conditions such as an acidic environment.

Further studies are needed to investigate whether unknown cofactors or environmental
conditions are required to maintain the strain-specific conformations in subsequent rounds. On the other hand, this hypothesis also explains why prion infectivity was lost in the fifth round of RT-QUIC, as nonspecific rPrP-fibrils generated during the serial RT-QUIC would be non-infectious. Although there remains the question as to what exactly are the conformational differences between the non-infectious and infectious forms of rPrP-fibrils, the lack of cofactor molecules such as SDS and synthetic PE in the RT-QUIC might enhance the amplification of nonspecific rPrP-fibrils lacking prion infectivity. Moreover, the facts that prion infectivity is sometimes too low to be detected and, more frequently, declines in the serial rPrP-PMCA (24, 25) or BH-PMCA (51-53) are consistent with the hypothesis.

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References


Deleault NR, Piro JR, Walsh DJ, Wang F, Ma J, Geoghegan JC, Supattapone S. 2012. Isolation of phosphatidylethanolamine as a solitary cofactor for prion


30. Atarashi R, Moore RA, Sim VL, Hughson AG, Dorward DW, Onwubiko HA,


Real time quaking-induced conversion analysis of cerebrospinal fluid in sporadic


FIGURE LEGENDS

FIGURE 1. The formation of rPrP-fibrils in RT-QUIC reactions. (A) The formation of rPrP-fibrils in the presence of diluted Chandler- or 22L-BH containing 100 pg of PrPSc, a comparable amount of NBH, or in the absence of seed (No-seeded) was monitored by ThT fluorescence. The graphs depict a representative of the RT-QUIC reactions. No-seeded reactions were performed at two different concentrations (100 or 50 µg/ml) of rPrP. (B) The QUIC reactions were digested with PK and immunoblotted using polyclonal anti-PrP antibody R20 (epitope located at mouse PrP amino acids 218-231) or ICSM35 (epitope 93-102). For comparison, 1st-rPrP-fibCh (50 ng of total rPrP) without PK digestion (PK (−)) is shown. Molecular mass markers are indicated in kilodaltons (kDa) on the left side of each panel. (C) Samples (1st-rPrP-fibCh, 1st-rPrP-fib22L and rPrP-fibpon) were examined with transmission electron microscopy (TEM). Scale bar, 100 nm. (D) The bar graph shows the length and width of rPrP-fibpon, 1st-rPrP-fibCh and 1st-rPrP-fib22L. The results are the mean ± SD of thirty rPrP-fibrils each. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Tukey-Kramer test. *, p < 0.01.
FIGURE 2. The formation of PrPSc-seeded rPrP-fibrils in the second (2nd-rPrP-fibSc) and fifth rounds (5th-rPrP-fibSc) of RT-QUIC. (A) The former reactions were diluted 100-fold into fresh rPrP between each round. The reaction buffer contained 300 mM NaCl, 50 mM HEPES (pH 7.5) and 10 μM ThT. The rPrP concentration was 100 μg/ml. (B) Transmission electron microscopic analysis of PrPSc-seeded rPrP-fibrils generated in the second, and fifth rounds of RT-QUIC. Scale bar, 100 nm.

FIGURE 3. Silver staining and Western blot analysis of purified PrPSc. (A) The purified PrPSc samples (P) were examined by silver-stained SDS-PAGE gel analysis (left panel). For comparison, the electrophoretic pattern of prion-infected brain homogenates (BH) containing 100 μg total protein digested with PK (20 μg/ml, 37 °C for 1h) is shown (left panel). The purified PrPSc samples (P) were immunoblotted with polyclonal anti-PrP antibody M20 (right panel). Molecular mass markers (M) are indicated in kilodaltons (kDa) on the left side of each panel. (B) Electron microscopy analysis of purified 22L-PrPSc (left panel) and Chandler-PrPSc (right panel). Scale bar, 100 nm.
FIGURE 4. FTIR spectroscopic characterization of rPrP-fibrils and purified PrPSc. (A) Second-derivative FTIR spectra are shown for purified PrPSc, 1st-rPrP-fibSc, 5th-rPrP-fibSc, spontaneous formation of rPrP-fibrils (rPrP-fibspont), and native rPrP. Overlaid spectra are from independent preparations. (B) FTIR spectra of rPrP-fibrils generated at pH 7.5 in the presence of low amount (1 pg) of PrPSc, and rPrP-fibrils generated at pH 4 in the presence of 100 pg of PrPSc.

FIGURE 5. Conformational stability assay for PrPSc in BH and rPrP-fibrils. (A) Chandler- (upper panel) or 22L (lower panel)-infected BH was treated with 0 to 3.5 M GdnHCl and subjected to PK digestion. PrPSc was detected by R20 anti-PrP polyclonal antibody. The denaturation curves were plotted using Boltzmann curve fit (right panel). (B–D) PK-digested 1st-rPrP-fibSc (generated as in Fig. 1) and rPrP-fibspont (B), 2nd-rPrP-fibSc(C), or 5th-rPrP-fibSc (D) was analyzed by Western blotting following GdnHCl treatment (0 to 5M). The PK-resistant fragments of the rPrP-fibrils were detected by R20.
FIGURE 6. Bioassay of rPrP-fibrils in mice. (A) PrPSc in the brains of prion-affected mice inoculated with 1st-rPrP-fibCh or 1st-rPrP-fib22L was analyzed by Western blotting using anti-PrP antibody M20. M, Mock 1st-QUIC(Ch) or Mock 1st-QUIC(22L). (B) Strain-specific properties of PrPSc in the brains of 1st-rPrP-fibSc-inoculated mice were examined by a conformational stability assay with GdnHCl (0 to 3.5M). (C) Sections of the hippocampus (HI) and cerebellum (CE), stained with hematoxylin and eosin, from normal mice, 1st-rPrP-fibSc-inoculated mice, and Mock 1st-QUIC-inoculated mice at terminal stages are shown. Scale bar, 50 µm. (D) Lesion profiles of spongiform changes in the hippocampus (HI), cerebral cortex (Cx), thalamus (TH), pons (Po) and cerebellum (CE) were compared. Data are expressed as means ± SD (n=3). Statistical significance was determined using Mann-Whitney’s U test. **, p < 0.01; *, p < 0.05.
**TABLE 1.** Conformational stabilities of purified PrP\(^{Sc}\) and rPrP-fibrils

<table>
<thead>
<tr>
<th>Strain</th>
<th>Purified PrP(^{Sc})</th>
<th>1(^{st})</th>
<th>2(^{nd})</th>
<th>5(^{th})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chandler</td>
<td>3.3 ± 0.4 **</td>
<td>3.3 ± 0.1 *</td>
<td>3.7 ± 0.1</td>
<td>3.3 ± 0.3</td>
</tr>
<tr>
<td>22L</td>
<td>1.7 ± 0.3</td>
<td>2.3 ± 0.6</td>
<td>3.8 ± 0.2</td>
<td>3.5 ± 1.0</td>
</tr>
<tr>
<td>Spontaneous</td>
<td>&gt; 5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) The \([\text{GdnHCl}]_{1/2}\) values (mol/l) are means ± SD of three independent experiments. Statistical significance was determined using one-way ANOVA followed by student’s \(t\)-test. **, \(p < 0.01\); *, \(p < 0.05\) (compared with 22L).
### TABLE 2. Bioassay for rPrP-fibrils generated in QUIC reactions in wild-type mice

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Concentration of seed PrPSc (pg/μl)</th>
<th>Survival periods (dpi)</th>
<th>Mortality (no. dead/total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st-rPrP-fib&lt;sup&gt;Ch&lt;/sup&gt;</td>
<td>1</td>
<td>185.5 ± 4.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4/4</td>
</tr>
<tr>
<td>Mock 1&lt;sup&gt;st&lt;/sup&gt;-QUIC(Ch)</td>
<td>1</td>
<td>201, 220&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2/4</td>
</tr>
<tr>
<td>1st-rPrP-fib&lt;sup&gt;22L&lt;/sup&gt;</td>
<td>1</td>
<td>213.0 ± 8.9&lt;sup&gt;**d&lt;/sup&gt;</td>
<td>6/6</td>
</tr>
<tr>
<td>Mock 1&lt;sup&gt;st&lt;/sup&gt;-QUIC(22L)</td>
<td>1</td>
<td>333&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1/5</td>
</tr>
<tr>
<td>5th-rPrP-fib&lt;sup&gt;Ch&lt;/sup&gt;</td>
<td>1 × 10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>&gt; 660&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0/4</td>
</tr>
<tr>
<td>Mock 5th-QUIC(Ch)</td>
<td>1 × 10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>&gt; 660&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0/4</td>
</tr>
<tr>
<td>5th-rPrP-fib&lt;sup&gt;22L&lt;/sup&gt;</td>
<td>1 × 10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>&gt; 660&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0/6</td>
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<td>1 × 10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>&gt; 660&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0/6</td>
</tr>
<tr>
<td>rPrP-fib&lt;sup&gt;spon&lt;/sup&gt;</td>
<td>0</td>
<td>&gt; 660&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0/6</td>
</tr>
</tbody>
</table>

**Second passage of 1<sup>st</sup>-rPrP-fib<sup>Ch</sup>**

**Second passage of Mock 1<sup>st</sup>-QUIC(Ch)**

**Second passage of 1<sup>st</sup>-rPrP-fib<sup>22L</sup>**

**Second passage of Mock 1<sup>st</sup>-QUIC(22L)**

<sup>a</sup> Mice were intracerebrally inoculated with 40 μl of each inoculum. For the second passage, 10% BH was used. Statistical significance was determined using Logrank test. ***, p < 0.01; *, p < 0.05** (compared with the controls).

<sup>b</sup> Days post-inoculation (dpi).

<sup>c</sup> After subjecting seed-only mixtures containing the same concentration of PrP<sup>Sc</sup> as 1<sup>st</sup>- or 5<sup>th</sup>-rPrP-fib<sup>Sc</sup> to a mock QUIC procedure, the same amount of rPrP was added. The solutions were inoculated into mice as controls for rPrP-fibrils.

<sup>d</sup> Numbers represent means ± SD.

<sup>e</sup> Numbers represent the survival periods of the TSE-positive mice. All non-symptomatic
mice were negative for PrP\textsuperscript{sc} at 660 dpi.

Numbers represent dpi when the experiment was ended.

A 201-dpi mouse was used.

A 333-dpi mouse was used.
Figure 2

A

2\textsuperscript{nd} round

22L

5\textsuperscript{th} round

Chandler

B

Chandler

22L
Figure 4

A

Chandler-PrP\textsuperscript{Sc}

22L-PrP\textsuperscript{Sc}

1\textsuperscript{st}-rPrP-fib\textsuperscript{Ch}

1\textsuperscript{st}-rPrP-fib\textsuperscript{22L}

5\textsuperscript{th}-rPrP-fib\textsuperscript{Ch}

5\textsuperscript{th}-rPrP-fib\textsuperscript{22L}

Native rPrP

rPrP-fib\textsuperscript{spon}

B

1 pg PrP\textsuperscript{Sc}-seeded at pH 7.5

100 pg PrP\textsuperscript{Sc}-seeded at pH 4

Ch

22L
**Figure 5**

**A**

PrP<sup>Sc</sup>

- Chandler
- 22L

GdnHCl (M)

0 1 1.5 2 2.5 3 3.5

**B**

1<sup>st</sup> round

- Chandler-seeded
- 22L-seeded
- Spontaneous

GdnHCl (M)

0 1 2 3 4 5

**C**

2<sup>nd</sup> round

- Chandler-seeded
- 22L-seeded

GdnHCl (M)

0 1 2 3 3.5 4 4.5 5

**D**

5<sup>th</sup> round

- Chandler-seeded
- 22L-seeded

GdnHCl (M)

0 1 2 3 4 5
Figure. 6

A

1st-rPrP-fib\textsuperscript{Ch} 1st-rPrP-fib\textsuperscript{22L}

(kDa) M 1 2 3 M 1 2 3

0 1 1.5 2 2.5 3 3.5

GdnHCl (M)

B

1st-rPrP-fib\textsuperscript{Ch} 1st-rPrP-fib\textsuperscript{22L}

0 1 1.5 2 2.5 3 3.5

GdnHCl (M)

C

HI CE

Normal

Mock 1st-QUIC (Ch)

1st-rPrP-fib\textsuperscript{Ch}

Mock 1st-QUIC (22L)

1st-rPrP-fib\textsuperscript{22L}

D

Mock 1st-QUIC

1st-rPrP-fib\textsuperscript{Sc}

Second passage

Chandler

Vacuolation score

Mock 1st-QUIC

1st-rPrP-fib\textsuperscript{Sc}

Second passage

Vacuolation score

22L