Recent advances in cell-free PrP^{Sc} amplification technique

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Abstract

The development of amplification technology for abnormal forms of prion protein *in vitro* has had a great impact on the field of prion research. This novel technology has generated new possibilities for understanding the molecular basis of prions and for developing an early diagnostic test for prion diseases. This review provides an overview of recent progress in cell-free PrP^{Sc} amplification techniques.

Cell-free conversion and PMCA

The main molecular event of prion diseases is the conversion of the normal protease-sensitive form of prion protein (PrP^{C} or PrP-sen) into pathological protease-resistant prion protein (PrP^{Sc} or PrP-res). According to the protein-only hypothesis, the continuous conversion of PrP^{C} to PrP^{Sc} in infected hosts is the mechanism of prion propagation. To further understand mechanistic details of the conversion, numerous attempts to develop cell-free systems have been made. "Cell-free conversion" was first demonstrated by Caughey and colleagues in 1994 [1]. They showed that largely purified PrP^{C} can be converted to the protease-resistant form only in the presence of purified brain-derived PrP^{Sc}. In cell-free conversion reactions, species and strain specificity was observed which positively correlates with *in vivo* transmission of prions [2]. However, the yield of cell-free conversion product was very limited, which made it difficult to determine if the newly synthesized PrP^{Sc} was infectious.

In order to overcome the low efficiency of cell-free conversion, Soto and colleagues developed a new method that can amplify PrP^{Sc} exponentially, designated protein misfolding cyclic amplification (PMCA) [3]. This method consists of repeated cycles of incubation and sonication. During the first step, prion-infected brain homogenate (a source of PrP^{Sc}) is incubated with excesses of normal brain homogenate (a source of PrP^{C}) to allow extension of PrP^{Sc} polymers. Subsequently, sonication is performed to break down large PrP^{Sc} polymers into smaller pieces to increase the number of seeds. Soto and colleagues also demonstrated that *in vitro* generation of PrP^{Sc} was accompanied by an increase of infectivity [4]. Although the finding greatly

strengthened the protein-only hypothesis, it was difficult to determine whether other molecules present in the brain homogenate are related to infectivity.

In an effort to clarify essential components of prion propagation in the PMCA, Supattapone and colleagues showed that PrP^{Sc} amplification can be achieved using PrP^{C} purified from brain tissue and polyanions, such as nucleic acids [5]. Furthermore, they discovered that infectivity as well as PrP^{Sc} generation could be induced spontaneously, even without PrP^{Sc} derived from prion-infected brain tissue [6]. These results provide the first evidence that infectious prions can be created by using PMCA reactions with noninfectious components.

Application of PMCA to early diagnostic tests for prion diseases

Another important application of the PMCA technique is the development of highly sensitive detection of PrP^{Sc}, the most reliable marker for prion infections. Detection of PrP^{Sc} in the preclinical stage of an affected host would lead to not only preventing spread of prion infection but also new opportunities for treating prion diseases; animal experiments have demonstrated that drug therapy is much more effective when initiated early in the incubation period [7, 8]. Using a hamster model infected by 263K strain, several rounds of successive PMCA enabled the ultrasensitive PrP^{Sc} detection in brain, blood and urine samples [9, 10]. Hence, PMCA is one of the most promising approaches for early diagnosis of prion diseases. However, there are still many technical problems in application of PMCA to routine analysis. First, it takes 3 weeks to achieve maximal sensitivity even using the hamster 263K strain [11]. Second, amplification levels in other species, including mice [12], cervids [13] and humans [14], have been much less than those in hamster to date. Third, PMCA requires the use of PrP^{C} derived from the same species brain tissues, which are often difficult to collect on a suitable scale.

PrP^{Sc} amplification technique using recombinant PrP

. Since the use of recombinant PrP (rPrP) as the amplification substrate could resolve some of the above problems, we sought to develop a new amplification method using recombinant hamster PrP (rHaPrP) and scrapie (263K strain) brain homogenate as a seed. It was necessary to find conditions under which rHaPrP could easily convert from protease-sensitive from (rPrP-sen) to protease-resistant form (rPrP-res), because rPrP was not an efficient substrate for conventional PMCA. We discovered that the conversion was greatly enhanced in the presence of low concentration (0.05-0.1%) of SDS and periodic sonication can amplify rPrP-res generation in an exponential manner

similar to conventional PMCA using brain-derived PrP^{C} [15] (Fig. 1). We named this method rPrP-PMCA. Although spontaneous formation of rPrP-res (termed rPrP-$res^{(spon)}$) was detected after many cycles of rPrP-PMCA, PrP^{Sc}–seeded rPrP-res (termed rPrP-$res^{(Sc)}$) and rPrP-$res^{(spon)}$ differed in their susceptibility to proteinase K (PK) digestion (Fig. 2A), thereby allowing clear and consistent discrimination between them. Furthermore, Fourier transform infrared spectroscopy (FTIR) analysis provided further evidence that they differ in conformation (Fig. 2B). Interestingly, when we examined the effects of dual seeding of rPrP-PMCA reactions with both rPrP-$res^{(spon)}$ and rPrP-$res^{(Sc)}$ using various seed ratios, competition between the two types of seeds was observed. This result indicates that the two fibrils are mutually exclusive and compete for rPrP-sen as a substrate, raising the possibility of molecular basis for *in vivo* competition/interference between prion strains.

The minimum amount of PrP^{Sc} detectable by rPrP-PMCA was around ~50 ag (5×10^{-17} g), or ~1,000 molecules, and it took only 2 days (2 rounds of rPrP-PMCA) to reach this detection limit [15]. This rapid amplification is probably due to the higher concentration of rPrP-sen relative to that of PrP^{C} in brain homogenates and is a great advantage of rPrP-PMCA, when compared with conventional PMCA. Moreover, this assay could discriminate between scrapie-infected and normal hamsters using cerebral spinal fluid (CSF).

However, we observed that the efficiency of amplification, especially in low seed amount, was influenced by tube position, tube construction, probe age, and bath volume of cuphorn type sonicator. This is most likely due to varied delivery of vibrational energy to the samples. Although most of these variations could be overcome with sufficient experience and equipment, we developed a new PrP^{Sc} amplification assay without sonication to circumvent such problems associated with the PMCA and rPrP-PMCA methods. This assay, designated QUIC (quaking-induced conversion), uses rPrP-sen as a substrate and intermittent automated tube shaking which can be performed more easily than sonication [16] (Fig 1). The sensitivity and the amplification speed in QUIC were equivalent to those in rPrP-PMCA, and QUIC reactions also discriminated between normal and scrapie-infected hamster CSF samples as did rPrP-PMCA. Another merit is that the emergence of rPrP-$res^{(spon)}$ in QUIC was much later than that in rPrP-PMCA. These characteristics of QUIC can reduce the risk of false-positive reactions and makes it possible to use easier and faster detection methods, such as thioflavin T (ThT) fluorescence dye that can be used to monitor amyloid formation. Others have shown that rPrP denatured by guanidine hydrochloride can form amyloid fibrils when seeded with partially purified PrP^{Sc} under continuous shaking, and the

amyloid formation can be detected using ThT fluorescence [17]. However, none of these assays have, thus far, been reported to achieve ultrasensitive PrP^{Sc} detection in readily accessible human specimens, such as CSF and blood from patients with human prion diseases.

In conclusion, striking progress has been made in cell-free PrP^{Sc} amplification technique, but further studies are needed to establish a practical test for an early and specific diagnosis of prion diseases. Additionally, these amplification technologies will facilitate future studies of the molecular basis of prion diseases, including the structure, toxic mechanism, and generation process of prions.

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Figure legend

Fig.1 Model of rPrP-PMCA or QUIC. In contrast to brain-derived PrP^{C}, partial unfolding of rPrP-sen is essential for efficient conversion to rPrP-res. The reason for this remains to be determined. Sonication or shaking causes fragmentation of rPrP-res aggregates. These cycles can be repeated for a desired number.

Fig.2 Comparisons between brain-derived PrP^{Sc}-seeded rPrP-res (rPrP-$res^{(Sc)}$) and unseeded rPrP-res (rPrP-$res^{(spon)}$). (This figure was modified from reference [15]). (A) PrP^{Sc}-seeded and unseeded products of rPrP-PMCA reactions were digested with PK and analyzed by immunoblot with R20 anti-PrP antibody. (B) FTIR spectroscopy of rPrP-$res^{(Sc)}$ and rPrP-$res^{(spon)}$**.** The spectrum of the rHaPrP-sen substrate is shown for comparison. Overlaid spectra are from independent preparations. As expected, rHaPrP-sen had an absorbance maximum at ~1652 cm^{-1}, consistent with prominent α-helical and/or disordered secondary structures. In contrast, both rPrP-$res^{(Sc)}$ and rPrP-$res^{(spon)}$ displayed prominent bands at lower wavenumbers (1615-1628 cm^{-1}), indicating higher proportions of β-sheet. However, the location of the bands differed between the two types of rHaPrP-res. The rPrP-$res^{(Sc)}$ had maxima at 1628 and 1615 cm^{-1}, whereas rPrP-$res^{(spon)}$ peaked at 1625 cm^{-1}. These spectral differences could be due to differences in conformation, PK-resistant polypeptide chain length, or both. Precise assignments of these bands are uncertain, but the 1664 cm^{-1} band is often associated with turns, and the 1659 and 1647 cm^{-1} bands with loops or helices, and disordered structures, respectively.

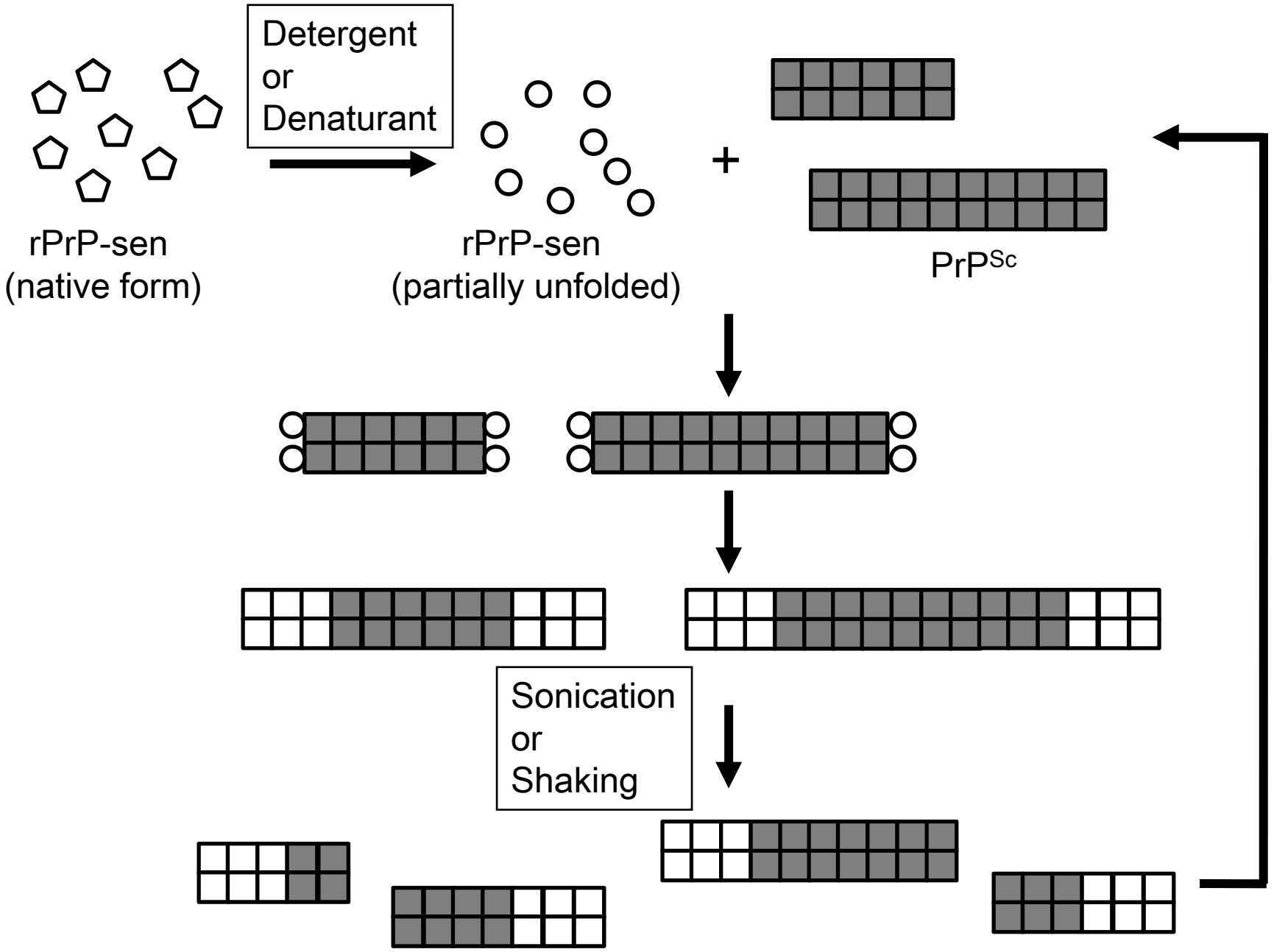
Detergent
or
Denaturant
rPrP-sen
(native form)
rPrP-sen
(partially unfolded)
+
PrPSc
Sonication
or
Shaking

Fig. 1

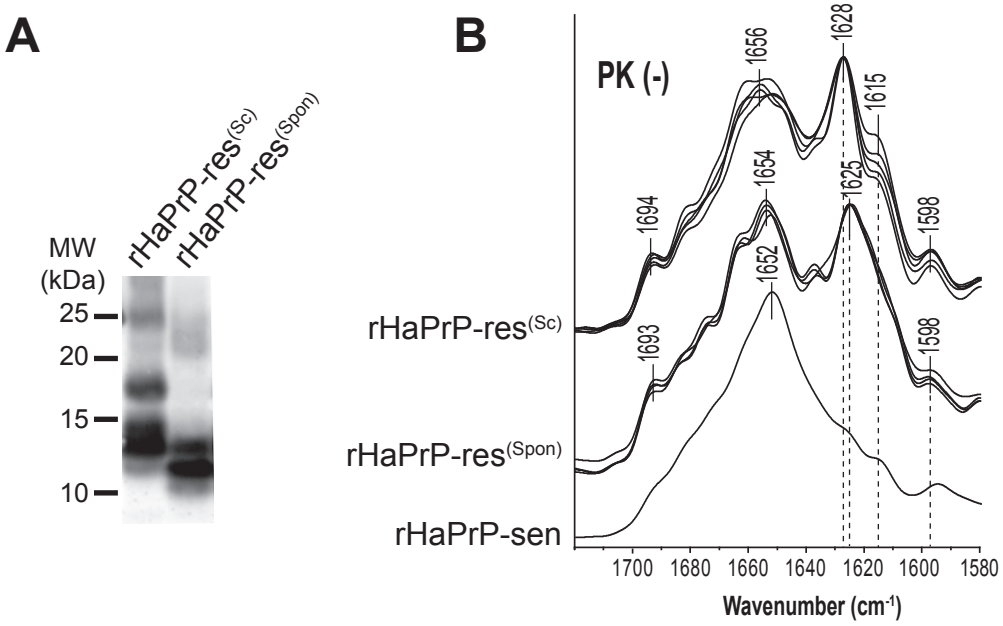
A
MW
(kDa)
25
20
15
10
rHaPrP-res(Sc)
rHaPrP-res(Spon)
B
PK (-)
1656
1628
1615
1694
1654
1625
1598
1652
1693
1598
rHaPrP-res(Sc)
rHaPrP-res(Spon)
rHaPrP-sen
1700 1680 1660 1640 1620 1600 1580
Wavenumber (cm-1)

Fig. 2