Chapter 6
Protein Misfolding Cyclic Amplification

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Abstract Prion diseases are caused by a conformational conversion of the cellular prion protein (PrP^{C}) to a pathological conformer (PrP^{Sc}). The "prion-only" hypothesis suggests that PrP^{Sc} is the infectious agent that propagates the disease acting as a template for the conversion of PrP^{C}. In 2001, we developed a novel in vitro technique, called Protein misfolding cyclic amplification (PMCA), which mimics this pathological process in an accelerated way. Thereby, minimal amount of PrP^{Sc} can be amplified to several millions fold, providing an important tool for diagnosis and investigation of prion biology, and the molecular mechanism of prion conversion. PMCA also offers a great platform for the study and amplification of the protein misfolding process associated with other neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases.

Keywords Prion diseases • Transmissible spongiform encephalopathies • Protein misfolding cyclic amplification • PMCA • Prion transmission • Prion decontamination procedures

6.1 PMCA: A Great Tool to Study Prion Biology

Prion diseases, or transmissible spongiform encephalopathies (TSEs), are a group of fatal disorders that affect both humans and animals. Prions are the proteinaceous infectious agent that is responsible for TSEs. Prions replicate through a nucleation-dependent process which is characterized by a long and silent incubation period followed by a rapid clinical phase. Thereby, a minute quantity of the pathological

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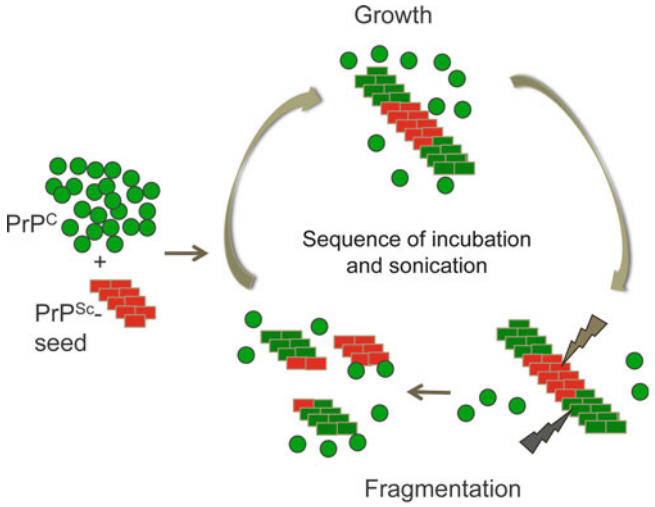

Fig. 6.1 Schematic representation of the PMCA principle. PMCA offers the chance to amplify minute quantities of PrP^{Sc} to a detectable level. In a cyclic manner consisting of two phases (incubation and sonication), PrP^{Sc} seeds from a sample are amplified at expenses of an excess of PrP^{C}. During the incubation phase, polymers of PrP^{Sc} grow by incorporation of PrP^{C}. In the following sonication phase, the large polymers are fragmented to generate multiple smaller PrP^{Sc} seeds for further prion replication

prion protein (PrP^{Sc}) works as a template and induces the conformational conversion of the cellular prion protein (PrP^{C}) to the pathogenic isoform (Prusiner 1998).

In 2001, we described an efficient technique to reproduce prion replication in the test tube in an accelerated manner, which is called protein misfolding cyclic amplification (PMCA) (Saborio et al. 2001). PMCA consists of cycles of incubation and sonication of a sample containing small amounts of PrP^{Sc} in the presence of an excess of PrP^{C}. During the incubation step, PrP^{Sc} aggregates grow through recruitment and conversion of PrP^{C} molecules. The following sonication phase is responsible for fragmenting these polymers to create new PrP^{Sc} seeds, which can induce further conversion of the cellular prion protein (Saborio et al. 2001; Soto et al. 2002). This method allows the exponential amplification of PrP^{Sc} in a PCR-like manner, and can begin the reaction with the equivalent to a single molecule of PrP^{Sc}, which after amplification can give rise to billions of PrP^{Sc} molecules (Saa et al. 2006a). The principle of PMCA is schematically illustrated in Fig. 6.1.

In following years, PMCA was improved through automation and the development of serial PMCA (sPMCA) (Fig. 6.2). Thereby, an aliquot of a PMCA sample, already subjected to many cycles of incubation and sonication, was diluted into fresh uninfected brain homogenate and subsequently exposed to further PMCA cycles. In this way, minute amounts of PrP^{Sc} can be detected through autocatalytic in vitro amplification, while the original inoculum is continuously diluted (Bieschke et al. 2004; Castilla et al. 2005a). An additional improvement was the addition of Teflon beads, which increase the efficiency and reproducibility of prion amplification (Gonzalez-Montalban et al. 2011).

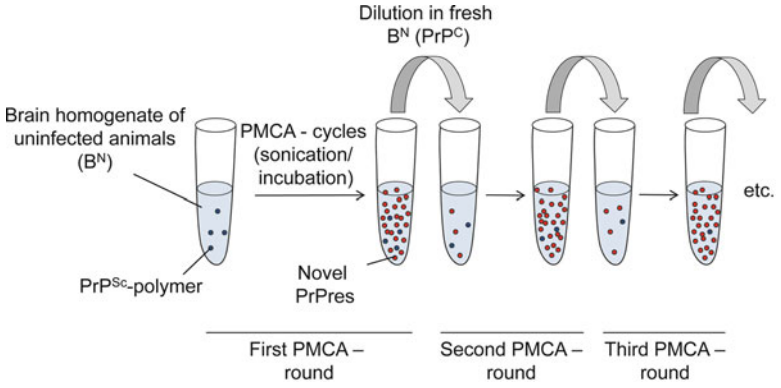

Fig. 6.2 Schematic design of serial PMCA. An aliquot of a PMCA sample, previously exposed to several PMCA cycles of incubation and sonication, is diluted in fresh brain homogenate and exposed to further PMCA cycles. Through sequences of serial PMCA rounds, the inoculum will be infinitely diluted and, in this way, prions can be maintained replicating indefinitively in vitro

Further experiments showed that the in vitro generated prions were fully infectious when injected into wild-type animals (Castilla et al. 2005a). They caused a similar disease with analog biochemical, biological, and structural properties observed in animals injected with brain derived PrP^{Sc} (Castilla et al. 2005a; 2008a; Weber et al. 2007). Studies of the components required to sustain PMCA amplification demonstrated the importance of cellular cofactors (e.g., nucleic acids and lipids) for efficient prion amplification (Deleault et al. 2003, 2007; Abid et al. 2010). Moreover, extensive PMCA cycling allows de novo formation of infectious prions mimicking the sporadic appearance of the disease (Deleault et al. 2007; Barria et al. 2009). In some of these cases, the prions produced through de novo creation in the test tube produced a new disease phenotype with unique clinical, neuropathological, and biochemical characteristics, never seen in nature (Barria et al. 2009).

An important development was the use of bacterially expressed recombinant prion protein (rPrP) as a substrate for PMCA (Atarashi et al. 2007). Wang and coworkers demonstrated that infectious prions can be generated from rPrP in the presence of synthetic lipids together with total RNA from normal mouse liver. When injected into wild-type mice, they caused a prion disease with similar incubation periods compared to naturally occurring prions (Wang et al. 2010). Recombinant PrP could also be labeled to perform structural studies of the prion protein.

The ability of PMCA to mimic the process of prion conversion in vitro provides great opportunities to analyze many aspects of prion biology, including (1) the biochemical mechanism of prion conversion and replication, (2) the species barrier and prion strain phenomena, (3) the potential role of cellular cofactors in PrP^C to PrP^{Sc} conversion, (4) the sensitive detection of prions for an early diagnosis of patients silently incubating the disease, (5) the evaluation of methods to remove and decontaminate prions, (6) the identification of prions in biological and environmental samples, and (7) the discovery and development of novel drugs to halt the prion conversion process.

6.2 PMCA Applications to Understand the Mechanism of Prion Transmission, Species Barrier and Strain Phenomena

Interspecies prion transmission is a process not well understood and limited by the so-called "species barrier" that corresponds to the ability of prions coming from one species to infect only a limited number of other species (Hill and Collinge 2004). This phenomenon is manifested as an incomplete attack rate and prolongation of the time to develop the disease in animals injected with infectious material from another species. The molecular basis of this event is not clear but convincing evidences indicate that the sequence of PrP controls this process; however the degree of the species barrier cannot be measured only by comparing the sequence of the proteins (Moore et al. 2005). The best way to investigate the species barrier is by infectivity experiments using animal models of the disease. However, these studies are costly and time-consuming because it is necessary to wait for several months or even years until the animals develop the clinical symptoms. Furthermore, the assessment of the species barrier for prion transmission to humans is compromised by the use of transgenic animal models expressing human PrP^{C}. PMCA can provide an in vitro alternative for studying the species barrier by combining PrP^{Sc} and PrP^{C} from different sources in distinct quantities. In this way, it is possible to quantitatively evaluate the efficiency of the conversion. Several studies confirmed that PMCA exhibits species specificity that faithfully reflects the same transmission barrier observed in animals (Castilla et al. 2008b; Green et al. 2008; Meyerett et al. 2008).

Transmission of sCJD between humans occurred through neurosurgical procedures as a consequence of using inappropriate techniques to sterilize instruments or devices that had been in contact with the brain tissue of sCJD infected individuals. Treatment with human-derived pituitary growth hormones or cornea or dura mater transplants, derived from infected recipients, also efficiently transmitted the disease (Brown et al. 2000). Conversely to vCJD, numerous studies have shown no evidence of human-to-human transmission of sCJD through the transfusion of blood or plasma, or the administration of plasma-derived therapeutic products (Operalski and Mosley 1995). Prions can also be transmitted from animals to humans. Epidemiological evidence suggests that among the animal TSEs, only BSE has been transmitted to humans through the consumption of contaminated beef products, generating the variant form of CJD (Will et al. 1996). Another concern is CWD, a disorder affecting mule deer and elk (Sigurdson and Aguzzi 2006) with high incidence in North America. CWD is highly transmissible within deer and elk populations. The mechanism of transmission is not well understood, but evidence supports the possibility that the disease is spread through direct animal-to-animal contact or as a result of indirect exposure to prions in the environment (e.g., in contaminated food and water sources). Transmission of CWD to humans cannot be excluded at this moment and transmissibility studies have been performed in many species to predict the spreading of the disease (e.g., in consequence of the consumption of CWD infected meat) (Sigurdson and Aguzzi 2006). In a recent study, we showed

that cervid PrP^{Sc} can induce the conversion of human PrP^{C}, but only after the CWD prion strain has been stabilized by successive passages in vitro or in vivo (Barria et al. 2011). Interestingly, the newly generated human PrP^{Sc} exhibits a distinct biochemical pattern that differs from any of the currently known forms of human PrP^{Sc}. These findings imply that CWD prions have the potential to infect humans, and that this ability depends on CWD strain adaptation.

An intriguing feature of prions that has been often used against the prion hypothesis is the existence of prion strains (Soto 2011). Nearly all TSEs are known to exhibit various strains characterized by different incubation periods, clinical features, and neuropathology (Morales et al. 2007). In traditional infectious diseases, different strains generally arise from mutations or polymorphisms in the genetic makeup of the infectious agent. To reconcile the infectious agent composed exclusively of a protein with the strain phenomenon, it has been proposed that PrPSc obtained from different prion strains has slightly different conformation or aggregation states that can faithfully replicate at the expense of the host PrPC (Bessen et al. 1995; Telling et al. 1996; Safar et al. 1998). Various reports have shown that PMCA allows the faithful replication of prion strains in many different species of prions, indicating that all the elements required for strain determination are enciphered in the folding of PrP^{Sc} (Castilla et al. 2008a; Jones et al. 2009; Shikiya and Bartz 2011).

6.3 PMCA Applications in Prion Detection and Diagnosis

Clinical diagnosis of definite CJD can only be made postmortem by histological analysis of spongiform changes and accumulation of PrP^{Sc} in the brain (Soto 2004). Since presymptomatic detection of sCJD or variant CJD (vCJD) in living people is currently not possible, it is important to develop an objective and sensitive test which has the potential to identify infected individuals at presymptomatic stages of the disease.

To date, PrP^{Sc} represents the main component of the infectious agent and is the only disease-specific marker for CJD (Prusiner 1998; Brown et al. 2001; Soto 2004). It is abundant in the brain at late stage of the disease, while minute amount are present in peripheral tissues and biological fluids, such as lymphoid organs, cerebrospinal fluid (CSF), urine, and blood (Aguzzi 2000; Brown et al. 2001; Wadsworth et al. 2001; Gonzalez-Romero et al. 2008). The latter two fluids could be the best candidates for routine noninvasive diagnostic tests, but there is no validated method to detect PrP^{Sc} in these biological fluids (Soto 2004). In this regard, we and others recently reported that PMCA enables detection of PrP^{Sc} in samples of blood and/or urine from prion-infected hamsters, mice, sheep, and cervids (Castilla et al. 2005b; Gonzalez-Romero et al. 2008; Thorne and Terry 2008; Haley et al. 2009; Tattum et al. 2010). We also showed that PrP^{Sc} can be detected during the presymptomatic phase of the disease in blood (Saa et al. 2006b). These results are extremely important since it has been demonstrated that vCJD transmission occurred in patients after blood transfusion (Llewelyn, et al. 2004). Undetectable levels of PrP^{Sc} could be present in the blood of

individuals silently incubating vCJD who may never develop clinical symptoms but remaining asymptomatic carriers able to transmit the disease to other individuals (Bishop et al. 2006). This could be a big problem for public health, especially for individuals who routinely rely on the blood supply and blood therapies.

In contrast, it is completely unknown if patients with sCJD have PrP^{Sc} circulating in blood and urine. Considering that most urine proteins originate from blood, it is likely that during disease progression, PrP^{Sc} is released from brain or peripheral organs into the blood at low concentrations, which is then excreted into the urine. Additionally, using a modified PMCA procedure, detection of PrP^{Sc} in CSF of humans affected by sCJD has been recently reported (Atarashi et al. 2011).

These findings suggest that PMCA enables efficient, specific, and rapid detection of prions in a variety of samples, offering a high promise for developing a noninvasive early diagnosis of prion diseases. Serial PMCA have been also applied for the diagnosis of different forms of animal prion diseases, including scrapie in hamster, mice and sheep, BSE in cattle, and CWD in cervids. In particular, PMCA allowed detecting PrP^{Sc} in the brain of presymptomatic hamsters, enabling a clear identification of infected animals as early as two weeks after inoculation (Soto et al. 2005). We demonstrated as well the presence of PrP^{Sc} in an experimentally infected cow 32 months postinoculation, that did not show clinical signs and was negative by standard western blot analysis (Soto et al. 2005).

Early diagnosis is very important for improving therapeutic perspectives, as treatment should start in an early stage, before the appearance of clinical signs and the occurrence of irreversible brain alterations. In addition, it should be possible to screen blood banks, reduce the iatrogenic transmission of the disease, and identify populations at risk (Soto 2004).

6.4 PMCA Applications in Development of Drugs and Prion Decontamination Procedures

One of the best targets for TSE therapy is the inhibition and reversal of PrP^{C} to PrP^{Sc} conversion. In drug development, it is crucial to have a relevant and robust in vitro assay to screen compounds for activity before testing them in more time-consuming and expensive in vivo assays. PMCA represents a convenient biochemical tool to identify and evaluate the activity of drug candidates for TSE treatment, because it mimics in vitro the central pathogenic process of the disease. Inhibitors and promoters could be tested quickly in different contexts using even human and bovine prions, for which no prion-permissive culture cells have been generated. Also the simplicity of the method and the relatively rapid outcome are important features for this type of studies. Moreover, the fact that PMCA can be applied to prion conversion in different species provides the opportunity to validate the use in humans of drugs that have been evaluated in experimental animal models of the disease.

In a similar way, the efficacy of devices and procedures to remove infectious prions from biological or environmental samples can be investigated in a rapid and

efficient way using PMCA. The fact that PMCA enables to detect quantities of prions several order of magnitude smaller than infectivity bioassay makes PMCA more effective in studying prion removal procedures. Particularly useful for this type of application is the recent development of the quantitative PMCA technology which in addition to detect prions also permits to estimate the concentration of PrP^{Sc} present in the sample (Chen et al. 2010). Various recent articles have been published using PMCA to evaluate prion inactivation and removal from biological and environmental samples using diverse procedures (Morales et al. 2008; Pritzkow et al. 2011; Saunders et al. 2011; Ding et al. 2012).

6.5 Expanding PMCA Beyond Prion Diseases

As prion diseases, most of the neurodegenerative disorders like Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and Huntington's disease are thought to be caused by the brain accumulation of misfolded protein aggregates (Soto 2003). Protein misfolding and aggregation in other neurodegenerative diseases also follows a seeding-nucleation model involving the formation of similar intermediates and end products as in TSEs (Soto et al. 2006). Indeed, acceleration of protein aggregation by the addition of seeds has been convincingly reported in vitro for several proteins implicated in diverse diseases (Krebs et al. 2004). These theoretical considerations suggest that protein misfolding processes have the inherent ability to be transmissible (Soto et al. 2006). Strikingly, a series of recent and exciting reports, using cellular and/or animal models, have provided evidence suggesting that the transmission of protein misfolding by a prion-like mechanism might be at the heart of the most common neurodegenerative diseases (Meyer-Luehmann et al. 2006; Clavaguera et al. 2009; Ren et al. 2009; Frost et al. 2009; Munch et al. 2011; Morales et al. 2011; Mougenot et al. 2012). The similarities between TSEs and other neurodegenerative diseases in terms of their molecular mechanisms suggest that PMCA might be adapted to amplify the abnormal folding of these proteins as well. In very recent studies, we have been able to optimize PMCA for the detection of minute quantities of amyloid-beta misfolded oligomers in biological fluids of patients affected by Alzheimer's disease (Salvadores-Bersezio et al., manuscript submitted).

6.6 Concluding Remarks

PMCA was first published in a Nature article in 2001 (Saborio et al. 2001) and is today widely considered as a major breakthrough in science and technology. PMCA enabled for the first time to cyclically amplify the folding and biochemical properties of a protein in a manner conceptually analogous to the amplification of DNA by PCR. PMCA has enabled the generation infectious prions in vitro providing the

strongest proof in favor of the prion hypothesis and has permitted to detect for the first time infectious prions in blood, offering a great possibility for early diagnosis. Over the past 5 years, PMCA has become widely used and invaluable technique to study the diverse aspects of prions. The PMCA technology has been used by several groups to understand the molecular mechanism of prion replication, the cellular factors involved in prion propagation, the intriguing phenomena of prion strains and species barriers, to detect PrPSc in tissues and biological fluids, and to screen for inhibitors against prion replication. The impact of PMCA is not only restricted to replication of prions, because it represents a platform technology to amplify the process of protein misfolding of the many proteins in which this mechanism occurs.

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