Ultra-efficient Replication of Infectious Prions by Automated Protein Misfolding Cyclic Amplification

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Prions are the unconventional infectious agents responsible for transmissible spongiform encephalopathies, which appear to be composed mainly or exclusively of the misfolded prion protein (PrP\textsuperscript{Sc}). Prion replication involves the conversion of the normal prion protein (PrP\textsuperscript{C}) into the misfolded isoform, catalyzed by tiny quantities of PrP\textsuperscript{Sc} present in the infectious material. We have recently developed the protein misfolding cyclic amplification (PMCA) technology to sustain the autocatalytic replication of infectious prions \textit{in vitro}. Here we show that PMCA enables the specific and reproducible amplification of exceptionally minute quantities of PrP\textsuperscript{Sc}. Indeed, after seven rounds of PMCA, we were able to generate large amounts of PrP\textsuperscript{Sc} starting from a $1 \times 10^{-12}$ dilution of scrapie hamster brain, which contains the equivalent of ~26 molecules of protein monomers. According to recent data, this quantity is similar to the minimum number of molecules present in a single particle of infectious PrP\textsuperscript{Sc}, indicating that PMCA may enable detection of as little as one oligomeric PrP\textsuperscript{Sc} infectious particle. Interestingly, the \textit{in vitro} generated PrP\textsuperscript{Sc} was infectious when injected in wild-type hamsters, producing a disease identical to the one generated by inoculation of the brain infectious material. The unprecedented amplification efficiency of PMCA leads to a several billion-fold increase of sensitivity for PrP\textsuperscript{Sc} detection as compared with standard tests used to screen prion-infected cattle and at least 4000 times more sensitivity than the biochemical properties as follows: PrP\textsuperscript{C} is soluble in nondenaturing detergents; PrP\textsuperscript{Sc} is insoluble; PrP\textsuperscript{C} is readily digested by proteases; and PrP\textsuperscript{Sc} is partially resistant (7, 10).

To understand the mechanism of prion conversion, Caughey and co-workers (11) developed a cell-free conversion reaction that mimics in many aspects the prion replication process. One of the limitations of the cell-free conversion method is the relative low efficiency of PrP\textsuperscript{Sc} formation that diminishes its application to study the nature of the infectious agent and to attempt sensitive detection of the protein. More recently, we have developed a novel technique referred to as protein misfolding cyclic amplification (PMCA), in which it is possible to simulate prion replication in the test tube in an accelerated and efficient way (12). In a cyclic manner, conceptually analogous to PCR cycling, PrP\textsuperscript{Sc} is incubated with excess PrP\textsuperscript{C} to enlarge the PrP\textsuperscript{Sc} aggregates, which are then sonicated to generate multiple smaller units for the continued formation of new PrP\textsuperscript{Sc} (13). We have reported previously proof-of-concept experiments in which the technology was applied to replicate the misfolded protein from diverse species (12, 14). The newly generated protein exhibits the same biochemical, biological, and structural properties as brain-derived PrP\textsuperscript{Sc}, and strikingly, it is infectious to wild-type animals, producing a disease with characteristics that are identical to the illness produced by brain-isolated prions (15). The technology has been automated, leading to a dramatic increase in efficiency of amplification and its application for transmissible spongiform encephalopathy (TSEs) are neurodegenerative disorders of humans and animals. Historically, scrapie has been the most common TSE in animals, affecting sheep for over 200 years (1). The most recent and worrisome outbreak of an animal TSE disease is bovine spongiform encephalopathy (BSE) in cattle (2). BSE has important implications for human health, because the infectious agent can be transmitted to humans producing a new disease, termed variant Creutzfeldt-Jakob disease (3, 4). TSEs are characterized by an extremely long incubation period, followed by a brief and invariably fatal clinical disease (5). To date no therapy or early diagnosis is available.

The pathogen responsible for TSEs, called “prion,” is comprised mainly or exclusively of a misfolded protein named PrP\textsuperscript{Sc}, which is a post-translationally modified version of the normal prion protein (PrP\textsuperscript{C}) (6, 7). During the course of the disease, prions replicate by the autocatalytic conversion of PrP\textsuperscript{C} into PrP\textsuperscript{Sc}, triggered by the misfolded protein present in the infectious inoculum. The conversion seems to involve a conformational change whereby the α-helical content of the normal protein diminishes and the amount of β-sheet increases (8, 9).

To understand the mechanism of prion conversion, we have reported previously proof-of-concept experiments in which the technology was applied to replicate the misfolded protein from diverse species (12, 14). The newly generated protein exhibits the same biochemical, biological, and structural properties as brain-derived PrP\textsuperscript{Sc}, and strikingly, it is infectious to wild-type animals, producing a disease with characteristics that are identical to the illness produced by brain-isolated prions (15). The technology has been automated, leading to a dramatic increase in efficiency of amplification and its application...
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to detect PrPSc in blood of hamsters experimentally infected with scrapie (16).

In this study we describe in detail the methodological aspects for efficient PMCA amplification and the characterization of the technology for reproducibility, specificity, and sensitivity. We also study the minimum number of molecules needed to trigger amplification and the application of the PMCA technique to generate infectious material in vitro. Our results demonstrate that PMCA is capable of detecting as little as ~26 monomers of PrP, which, according to recent data on the minimal size of the infectious particle (17), would correspond to a single molecule of oligomeric infectious PrPSc. The technique is highly reproducible and specific to amplify PrPSc, because no signal is detected when no PrPSc inoculum is present. Finally, our results show that PMCA enables the increase of infectivity by around 20 million-fold, converting a sample that is not infectious into a highly infectious one. These data demonstrate that PMCA has a similar power of amplification as PCR techniques used to amplify DNA and have great promise for the development of highly sensitive detection of PrPSc and for understanding the molecular basis of prion replication.

EXPERIMENTAL PROCEDURES

Preparation of Tissue Homogenates—Healthy and sick animals were perfused with phosphate-buffered saline (PBS) plus 5 mM EDTA prior to harvesting the tissue. Ten percent brain homogenates (w/v) were prepared in conversion buffer (PBS containing 150 mM NaCl, 1.0% Triton X-100, 4 mM EDTA, and the Complete Protease Inhibitor Mixture from Roche Applied Science). The samples were clarified by a brief, low speed centrifugation (1500 rpm for 30 s) using an Eppendorf centrifuge, model 5414 (Hamburg, Germany). Dilutions of this brain homogenate were done in conversion buffer, and they are expressed in relation to the brain; for example, a 100-fold dilution is equivalent to a 1% brain homogenate.

In Vivo Infectivity Studies—Syrian Golden hamsters were used as an experimental model of scrapie. Animals were 4–6 weeks old at the time of inoculation. Anesthetized animals were injected intracerebrally stereotaxically in the right hippocampus with 1 μl of the sample or intraperitoneally with 200 μl of sample as described previously (15). The onset of clinical disease was measured by scoring the animals twice a week using the following scale: 1, normal animal; 2, mild behavioral abnormalities, including hyperactivity and hypersensitivity to noise; 3, moderate behavioral problems, including tremor of the head, ataxia, wobbling gait, head bobbing, irritability, and aggressiveness; 4, severe behavioral abnormalities, including all of the above plus jerks of the head and body and spontaneous backrolls; 5, terminal stage of the disease in which the animal lies in the cage and is no longer able to stand up. Animals scoring level 4 during 2 consecutive weeks were considered sick and were sacrificed to avoid excessive pain using exposure to carbonic dioxide. The scrapie infectious material used in these studies was titrated and 1 LD50 was considered sick and were sacrificed to avoid excessive pain.

Brains were extracted and analyzed biochemically and histologically. The right cerebral hemisphere was frozen and stored at −70 °C for biochemical examination of PrPSc using Western blot analysis as described below. The left hemisphere was fixed in 10% formaldehyde solution, cut into sections, and embedded in paraffin. Serial sections (6 μm thick) from each block were stained with hematoxylin-eosin, using standard protocols, or incubated with monoclonal antibodies recognizing PrP or the glial fibrillary acidic protein. Immunoreactions were developed using the peroxidase-antiperoxidase method, following the manufacturer’s specifications. Antibody specificity was verified by absorption. To compare the pattern of histopathological damage among animals, we calculated the lesion profile, using a variation of the method employed in mice (18). Briefly, the severity of vacuolation was scored in a scale from 0 to 5 in seven different brain areas, including medulla, cerebellum, superior colliculus, hippocampus, and cerebral cortex occipital, frontal, and lateral.

PMCA Procedure—Although the principle of PMCA remains the same as in our original publication (12), the system has been optimized and automated, thus enabling the routine processing of many more samples in the same amount of time while reaching a higher conversion efficiency. Aliquots of normal and scrapie brain homogenate prepared in conversion buffer were mixed and loaded onto 0.2-ml PCR tubes. Tubes were positioned on an adaptor placed on the plate holder of a microsonicator (Misonix model 3000, Farmingdale, NY) and programmed to perform cycles of 30 min of incubation at 37 °C followed by a 40-s pulse of sonication set at 60% potency. Samples were incubated without shaking and immersed in the water of the sonicator bath. and the entire microplate horn was kept inside an incubator at 37 °C. The detailed protocol, including troubleshooting, has been recently published elsewhere (19–21).

Protease K Digestion—Samples were incubated with 50 μg/ml PK for 60 min at 45 °C. The digestion was stopped by adding electrophoresis sample buffer. In each experiment it is important to have a negative control consisting of normal brain homogenate and to check that PK digestion of PrPC is complete to avoid confusion between undigested PrPC and the signal from the PK-resistant core of PrPSc. For this purpose special care has to be taken to observe the switch in the molecular weight after PK digestion, characteristic of bona fide PrPSc.

Western Blot—Proteins were fractionated by SDS-PAGE under reducing conditions, electroblotted into nitrocellulose membrane, and probed with 3F4 antibody (22) (Signet, Dedham, MA) diluted 1:5000 in PBS. The immunoreactive bands were visualized by enhanced chemiluminescence assay (Amersham Biosciences). Densitometric analysis was done by using a UVP Bioimaging system EC3 apparatus (Upland, CA). Statistical significance of the values was evaluated by Student’s t test.

ELISA—Twenty μl of serial dilutions of an infected hamster brain into 10% normal brain homogenate were treated with 50 μg/ml PK for 1 h at 45 °C and 450 rpm. The digestion was stopped with 50 mM phenylmethylsulfonyl fluoride. The digested samples were mixed with PBS up to 100 μl, loaded onto a 96-well ELISA plate (Maxisorp surface treatment), and incubated for 1 h at 37 °C. The plates were blocked with 5% BSA in PBS for 1 h at 37 °C, followed by incubation for 1 h with the monoclonal antibody 3F4 (Signet, Dedham, MA) diluted 1:3000.
in 1% BSA in PBS. The antibody was washed four times with 200 μl of PBS, 0.05% Tween 20, and samples were incubated with the polyclonal antibody anti-mouse IgG, conjugated with horseradish peroxidase (Amersham Biosciences), and diluted 1:1000 in 1% BSA in PBS. After four washes the samples were developed with ImmunoPure ABTS (Pierce) following the manufacturer’s directions.

PTA Precipitation—Twenty μl of serial dilutions of an infected hamster brain into 10% normal brain homogenate were first treated with benzonase (2.5 units/μl in PBS, 1 mM MgCl2) for 30 min at 37 °C with constant agitation. Thereafter, we added 4% PTA (prepared in 70 mM MgCl2, titrated with NaOH to pH 7.4) and incubated at 37 °C for 30 min with constant agitation. Samples were centrifuged at 15,800 × g for 30 min at 37 °C in an Eppendorf microcentrifuge. Pellet was resuspended in 20–30 μl of PBS containing 0.1% Sarkosyl. After treatment with PK, samples were analyzed by Western blot as described above.

PrPSc Quantitation—To estimate the quantity and the number of molecules of PrPSc in our samples, we analyzed several dilutions of scrapie brain homogenate by Western blot in the same gel as aliquots of known amounts of recombinant hamster PrP (supplemental Fig. 1A). The signal intensity was evaluated by densitometry, and the quantity of PrP in the sample was estimated by extrapolation of the calibration curve prepared with recombinant PrP (supplemental Fig. 1B). To minimize artifacts because of saturated or weak signal, several different dilutions were measured, and each dilution was analyzed in triplicate. To standardize the signal among the different blots, the densitometric data were expressed relative to the value of the signal of the same quantity of normal brain homogenate (without PK treatment). PrPSc quantitation was also confirmed by ELISA using recombinant PrP as standard. In this way we estimated the average concentration of PrPSc in the scrapie brain used in our studies to be 67 ng/μl. The number of molecules of PrPSc detected was estimated by mathematical calculation of the dilution used and the known concentration of PrPSc in the brain homogenate.

RESULTS

The PMCA Technology and Its Reproducibility—PMCA consists of cycles of accelerated prion replication. The basis for PMCA is the observation that prion replication follows a seeding-nucleation model in which oligomeric PrPSc in the infectious material converts PrPC by integrating monomeric proteins into the ends of the aggregate, inducing and stabilizing its misfolding (13, 23). PMCA is a cyclic process consisting of two phases. During the first phase the samples containing minute amounts of PrPSc and a large excess of PrPC were incubated to induce growing of PrPSc polymers. In the second phase the sample is sonicated to break down the polymers, multiplying the number of nuclei. In this way, after each cycle the number of “seeds” is increased in an exponential fashion. The number of cycles can be repeated as many times as needed to reach the amplification rate desired. For practical operation, the system has been automated by using a programmable plate sonicator, as described under “Experimental Procedures.” This improvement not only decreases processing time and allows for the routine processing of many more cycles than a single probe sonicator but also prevents loss of material. Cross-contamination is eliminated because there is no direct probe intrusion into the sample.

Reproducibility of amplification was measured by monitoring the PrPSc signal obtained before and after PMCA cycling under different experimental conditions. Equivalent samples containing a 10,000-fold dilution of scrapie brain into 10% healthy hamster brain homogenate were placed in distinct positions of the microplate sonicator and subjected to 48 PMCA cycles. The levels of amplification were studied by Western blot after PK digestion (Fig. 1A), and data were quantitated by densitometric analysis of the Western blot signal (Fig. 1B). Although some small variability was observed on the signal obtained in distinct wells, the differences were not significant and could not be attributed to a position effect, but rather they probably reflect some small experimental variability.

To analyze further the reproducibility of the procedure, equivalent samples containing a 10,000-fold dilution of scrapie brain homogenate into 10% healthy hamster brain homogenate were subjected to 48 PMCA cycles in experiments done on different days. Fig. 1C shows that at 7 distinct days the amplification efficiency was virtually the same. Again, densitometric analysis showed that the signal was not statistically different in the distinct experiments (Fig. 1D). For this experiment, the normal brain sample used on day 1 was freshly prepared, whereas for all the other days, frozen material was used. Therefore, no differences between fresh and frozen material were observed. However, it is important to note that brain substrate has to be frozen in aliquots to avoid repetitive freezing-thawing that decreases PMCA efficiency.

The influence of different, but equivalent, inocula on the conversion efficiency was studied by amplifying preparations of 10,000-fold diluted scrapie brain homogenate obtained from five distinct hamsters into the same substrate (Fig. 1E). After 48 PMCA cycles, a large and similar conversion of PrPSc into PrPSc was observed. A similar result was obtained when normal brain homogenate from five different hamsters was used as a substrate for the amplification of a unique PrPSc inoculum (Fig. 1G). However, densitometric analysis of the experiments shown in Fig. 1, E and G, indicate that in both cases one sample gave a statistically significant different level of amplification than the other four samples (Fig. 1, F and H, respectively). These differences are not because of changes in the levels of PrPSc or PrPC in the samples, which were not significantly different. These results suggest that perhaps individual variability on the expression of PrP or conversion factors may lead to changes on the extent of prion conversion in vitro. In none of the experiments shown in Fig. 1 was PrPSc detectable in samples containing the same material but kept frozen without amplification.

Specificity of PMCA Amplification—Specificity of cyclic amplification was evaluated in a blind study in which 10 brain samples of scrapie-affected hamsters and 11 samples of healthy animals were subjected to 48 PMCA cycles, and PrPSc was detected by Western blot analysis after PK digestion (Fig. 2, A and B). The results show that although 100% of the samples derived from sick animals (Fig. 2, A and B, lanes 1, 2, 3, 5, 7, 11,
13, 17, 19, and 21) were positive after PMCA, none of the samples coming from normal animals (lanes 4, 6, 8, 9, 10, 12, 14, 15, 16, 18, and 20) show any significant PrPSc signal. Of the 10 positive control samples, 7 corresponded to a 10,000-fold dilution of brain, 2 corresponded to a 50,000-fold dilution (Fig. 2, lanes 13 and 17 in), and 1 corresponded to a 100,000-fold dilution (Fig. 2A, lane 19). None of these 10 samples showed any PrPSc signal in Western blot without PMCA amplification (data not shown). The interpretation of the data is that, under the conditions used, PMCA leads to 100% specificity for PrPSc detection.

As demonstrated before, the amplification rate using PMCA depends upon the number of incubation/sonication cycles carried out (12). Thus, we decided to evaluate whether a PrPSc-like signal might appear on negative samples after many PMCA cycles. For this purpose, a 10% healthy hamster brain homogenate in the absence (negative control) or in the presence (positive control) of an aliquot of a 50,000-fold diluted scrapie brain was subjected to 24, 48, 96, or 144 PMCA cycles, and the PrPSc signal was detected by Western blotting after PK treatment. Densitometric analysis of three different blots obtained using the samples described in E, G, a single scrapie brain homogenate was diluted 10,000-fold into 10% solutions prepared from five different normal hamster brains (S1 through S5). Again 48 PMCA cycles were performed and PrPSc detected by Western blotting. Densitometric analysis of three different blots obtained using the samples described in G, F, frozen samples; A, amplified samples. NBH, normal brain homogenate. All samples were treated with PK before electrophoresis, except those in which −PK is indicated. Data were statistically analyzed by one-way analysis of variance and Student’s t test. *p < 0.05.

FIGURE 1. Reproducibility of automated PMCA. A, samples containing a 10,000-fold dilution of 263K scrapie hamster brain prepared in 10% normal hamster brain were either immediately frozen (F) or placed in 10 different positions of the plate holder and subjected to 48 PMCA cycles. Thereafter, samples were treated with PK and PrPSc reactivity analyzed by Western blotting. B, densitometric analysis of three different blots obtained using the samples described in A. C, equivalent samples prepared as described in A were subjected to 48 PMCA cycles performed at seven different times, and the extent of PrPSc formation was evaluated by Western blotting. D, densitometric analysis of three different blots obtained using the samples described in C. E, five different scrapie hamster brains (I1 through I5) were diluted 10,000-fold into the same 10% normal hamster brain homogenate and subjected to 48 PMCA cycles. PrPSc signal was detected by Western blotting after PK treatment. F, densitometric analysis of three different blots obtained using the samples described in E. G, a single scrapie brain homogenate was diluted 10,000-fold into 10% solutions prepared from five different normal hamster brains (S1 through S5). Again 48 PMCA cycles were performed and PrPSc detected by Western blotting. H, densitometric analysis of three different blots obtained using the samples described in G. F, frozen samples; A, amplified samples. NBH, normal brain homogenate. All samples were treated with PK before electrophoresis, except those in which −PK is indicated. Data were statistically analyzed by one-way analysis of variance and Student’s t test. *p < 0.05.
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In order to test the sensitivity of PMCA, we have diluted 10,000-fold into 10% normal brain homogenate. For all scrapie-infected samples, except for the ones in lanes 13, 17, and 19, the brain was diluted 10,000-fold into 10% normal brain homogenate. For lanes 13 and 17, the inoculum was diluted 50,000-fold and for lane 19, the brain was diluted 100,000-fold. After amplification, the samples were treated with PK and analyzed by Western blotting. As shown in Fig. 2, we were able to detect PrPSc up to a 5 × 10⁻¹⁰ dilution of scrapie hamster brain, to reach an increase of sensitivity of around 10 million-fold (16). To estimate the minimum number of molecules of PrPSc that our technology can detect in a given sample, we diluted a scrapie brain homogenate 1 × 10⁻¹²-fold into conversion buffer and subjected this material to saPMCA. According to our estimations, the PrPSc concentration in the scrapie-infected brain used for these studies was ~67 ng/μl (see “Experimental Procedures” and supplemental Fig. 1). This result indicates that a 1 × 10⁻¹²-fold dilution should contain ~6.7 × 10⁻²⁰ g/μl or 1.3 molecules of PrPSc monomer per μl. Because in our experiments we use a volume of 20 μl, the sample tested contains ~26 molecules of monomeric PrPSc. Strikingly, after five rounds of saPMCA, we were able to detect a signal in one of the four replicates used, and after seven rounds of amplification, we detected a signal in three of the four replicates (Fig. 3). Importantly, no amplified product was detected when a 10⁻¹⁴-fold dilution of brain was used (a sample that should contain no molecules of PrPSc) or in any of the control samples in which no PrPSc was present (Fig. 3). No signal was detected either in a 10⁻¹³-fold dilution (data not shown).

The serious consequences of the BSE epidemics and the increasing concern regarding the iatrogenic transmission of variant Creutzfeldt-Jakob disease have motivated the development of several biochemical methods to detect PrPSc. Several tests have been approved by the European community and are

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**FIGURE 2. Specificity of PrPSc detection after PMCA.** A blind study in which aliquots from 11 different normal brain samples and 10 distinct diluted scrapie-infected brain samples were subjected to 48 PMCA cycles to attempt detection of PrPSc. For all scrapie-infected samples, except for the ones in lanes 13, 17, and 19, the brain was diluted 10,000-fold into 10% normal brain homogenate. For lanes 13 and 17, the inoculum was diluted 50,000-fold and for lane 19, the brain was diluted 100,000-fold. After amplification, the samples were treated with PK and analyzed by Western blotting. B, densitometric analysis of three different blots from the samples shown in A, C, samples from healthy (denoted −PrPSc inoculum) or scrapie-infected (denoted +PrPSc inoculum) brain were diluted 50,000 into 10% normal brain homogenate and subjected to 0, 24, 48, 96, or 144 PMCA cycles, and PrPSc signal was analyzed by Western blotting. D, brains from healthy hamsters or from animals infected with 263K scrapie were diluted 10⁵-fold into a 10% normal hamster brain homogenate. Samples were subjected to 48 PMCA cycles. The amplified material was diluted 10-fold into normal brain homogenate and amplified again. This procedure was repeated several times to reach a 10⁻¹³ dilution of initial material. The figure shows the PrPSc signal obtained in Western blots of only some of the dilutions, representative of all the results obtained. NBH, normal brain homogenate. All samples were treated with PK before electrophoresis, except those in which −PK is indicated.

diluting the material to refresh the substrate. We have shown that PrPSc can be kept replicating indefinitely in vitro by several rounds of successive PMCA (15) and that serial PMCA enables the ultrasensitive detection of PrPSc in brain and blood samples (16). Brains from healthy hamsters and from animals infected with the 263K scrapie strain were diluted 10⁻⁵-fold into a 10% normal hamster brain homogenate. Samples were subjected to 48 PMCA cycles. After this first round of PMCA, a small aliquot of the amplified samples was taken and diluted 10-fold into more normal brain homogenate. These samples were again amplified by 48 PMCA cycles. This procedure was repeated several times, and PrPSc generation was determined by Western blot analysis after PK digestion. As shown in Fig. 2D, 10 rounds of PMCA to reach a final dilution of the original brain equivalent to 10⁻¹³ led to continuous formation of PrPSc only when the initial inoculum was derived from scrapie-infected animals. No PrPSc was ever detected in the absence of PrPSc inoculum, indicating that the system retains high specificity, even after 480 PMCA cycles. These same samples were used for further amplification, and after a dilution of more than 10⁻⁶³ of initial inoculum a robust and continuous amplification was observed in samples containing PrPSc. When many additional rounds of saPMCA were performed, we observed a scatter appearance of a protease-resistant band identical to PrPSc even in samples without PrPSc inoculum (data not shown). Spontaneous generation of PrPSc was seen mainly when more than 10 rounds of PMCA were done. At present we do not know whether this newly generated PrPSc is the result of cross-contamination or the de novo generation of PrPSc. More experiments need to be performed to analyze the reproducibility of this observation and to distinguish between these two possibilities. In case we ruled out the possibility of contamination, these findings would indicate that PMCA can amplify a low frequency event in which PrPSc spontaneously converts into PrPSc. This could be the basis for the origin of sporadic prion diseases.

Sensitivity of PMCA and Minimum Number of Molecules Detected after Amplification—We have recently reported that sensitivity of detection after 140 cycles of PMCA was increased by around 6600-fold (16). We also reported previously that to further increase replication efficiency, we needed to refresh the substrate periodically using a methodology we termed serial automated PMCA (saPMCA) (16). By performing two rounds of saPMCA, we were able to detect PrPSc up to a 5 × 10⁻¹⁰ dilution of scrapie hamster brain, to reach an increase of sensitivity of around 10 million-fold (16). To estimate the minimum number of molecules of PrPSc that our technology can detect in a given sample, we diluted a scrapie brain homogenate 1 × 10⁻¹²-fold into conversion buffer and subjected this material to saPMCA. According to our estimations, the PrPSc concentration in the scrapie-infected brain used for these studies was ~67 ng/μl (see “Experimental Procedures” and supplemental Fig. 1). This result indicates that a 1 × 10⁻¹²-fold dilution should contain ~6.7 × 10⁻²⁰ g/μl or 1.3 molecules of PrPSc monomer per μl. Because in our experiments we use a volume of 20 μl, the sample tested contains ~26 molecules of monomeric PrPSc. Strikingly, after five rounds of saPMCA, we were able to detect a signal in one of the four replicates used, and after seven rounds of amplification, we detected a signal in three of the four replicates (Fig. 3). Importantly, no amplified product was detected when a 10⁻¹⁴-fold dilution of brain was used (a sample that should contain no molecules of PrPSc) or in any of the control samples in which no PrPSc was present (Fig. 3). No signal was detected either in a 10⁻¹³-fold dilution (data not shown).

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Comparison of the sensitivity of several methods to detect PrPSc

| Assay                        | Maximum dilution detected | Minimum PrP quantity detected | Minimum no. of PrP molecules | Increase in sensitivity |
|-----------------------------|---------------------------|--------------------------------|----------------------------|------------------------|
| Standard Western blot       | $3.0 \times 10^{-2}$      | $4.0 \text{ ng}$               | $8.0 \times 10^{10}$        | $1$                    |
| ELISA                       | $3.7 \times 10^{-3}$      | $0.5 \text{ ng}$               | $1.0 \times 10^{10}$        | $8$                    |
| Phosphotungstic acid precip  | $6.0 \times 10^{-5}$      | $80 \text{ pg}$               | $1.6 \times 10^{9}$         | $50$                   |
| Conformation dependent immunoassay | $5.0 \times 10^{-5}$ | $67 \text{ pg}$               | $1.3 \times 10^{9}$         | $60$                   |
| Animal bioassay             | $2.0 \times 10^{-9}$      | $3.2 \times 10^{7}$           | $725,000$                   | $2,500$                |
| One round of PMCA           | $1.2 \times 10^{-10}$     | $1.6 \text{ pg}$              | $1.3 \times 10^{8}$         | $6,000,000$            |
| Two rounds of PMCA          | $5.0 \times 10^{-10}$     | $0.7 \text{ fg}$              | $1.3 \times 10^{8}$         | $6,000,000$            |
| Seven rounds of PMCA        | $1.0 \times 10^{-12}$     | $1.3 \text{ ag}$              | $26$                        | $3,000,000,000$        |

* The maximum dilution detected corresponds to the last dilution of 263K scrapie brain in which PrPSc is detectable.
* The minimum quantity of PrPSc detected in a brain sample volume of 20 μl.
* The number of PrP molecules detected in a 20 μl sample volume was estimated as described in supplemental Fig. 1 by comparison with recombinant PrP.
* The increase of sensitivity is expressed in relation to the standard Western blot assay using 3F4 antibody.
* The data for the conformation-dependent immunoassay were taken from the literature, whereas all the others were experimentally calculated.
* The data for PMCA correspond to the average obtained in three different experiments using 100 PMCA cycles in each round.
infectivity studies demonstrate that two and seven rounds of saPMCA are >8 and >4000 times more sensitive than the most efficient animal bioassay, respectively (Table 1).

Generation of Infectivity in Vitro from Sub-infectious Quantities of PrP<sup>Sc</sup>—As described in Fig. 3, we were able to generate large quantities of PrP<sup>Sc</sup> from a very high dilution (1 × 10<sup>-13</sup>) of hamster scrapie brain. Mathematical calculation estimates that such dilution contains the equivalent of ~26 molecules of PrP monomers, which are at least 3–4 orders of magnitude below the minimum quantity of PrP<sup>Sc</sup> needed to produce infectivity in some animals at very long times after inoculation. To study whether the in vitro generation of PrP<sup>Sc</sup> was associated with an increase of infectivity, we inoculated intraperitoneally the sample obtained after seven rounds of amplification starting with 1 × 10<sup>-12</sup> dilution (Fig. 3, sample S<sub>j</sub>). All six wild-type hamsters inoculated showed typical signs of 263K scrapie disease and were sacrificed at an average of 299.6 ± 20.5 days post-inoculation. In our experience with this animal model, this incubation time is similar to that obtained with a 5 × 10<sup>-8</sup> dilution of scrapie infected brain inoculated by the intraperitoneal route. Therefore, seven rounds of PMCA produced a 2 × 10<sup>12</sup> increase of infectivity. As expected none of the control animals inoculated with the same dilution without amplification developed the disease even after 500 days post-inoculation.

The clinical signs observed in animals inoculated with the in vitro amplified samples were identical to those of the animals treated with infectious brain material and included hyperactivity, motor impairments, head wobbling, muscle weakness, and weight loss. Brain samples from these animals contain a large quantity of protease-resistant PrP<sup>Sc</sup>, which has an identical glycosylation profile to the protein observed in brain-inoculated animals (Fig. 4A). Conversely, no protease-resistant protein was detected in the brain of negative control animals. Histological analysis showed typical brain spongiform degeneration, PrP accumulation, and astrogliosis (Fig. 4B). No differences in the lesion pattern profile were observed compared with animals inoculated by brain infectious material. These findings suggest that PrP<sup>Sc</sup> generated in vitro corresponds to the same strain of 263K used to trigger amplification.

**DISCUSSION**

Our results indicate that PMCA is able to amplify exceptionally small quantities of PrP<sup>Sc</sup> in a very specific and reproducible manner. Indeed, by using several successive rounds of PMCA, our data demonstrate that we are able to induce the conversion of PrP<sup>C</sup> with as little as the equivalent to ~26 molecules of PrP<sup>Sc</sup> monomers. Recent data have shown that the minimum size of the particle able to sustain infectivity and to induce the cell-free conversion of PrP<sup>C</sup> into PrP<sup>Sc</sup> contains between 14 and 28 molecules of PrP monomers (17). Taken together, this suggests that saPMCA can amplify a single particle of oligomeric infectious PrP<sup>Sc</sup>. This unprecedented amplification efficiency is comparable only to the effectiveness of PCR amplification of DNA (34). Moreover, although at these levels of amplification PCR often results in artifactual amplification products, we rarely see a false-positive using PMCA.

The applications of such a powerful amplification technology are many and impact various fields. A particularly important application is on the development of a highly sensitive biochemical detection of PrP<sup>Sc</sup>, which constitutes the best surrogate marker for TSE diagnosis (24). A comparison of saPMCA with the sensitivity of some of the current methodologies used for PrP<sup>Sc</sup> detection (Table 1) showed that our technology is several millions or even billions of times more sensitive than the standard Western blot or ELISA-based assays currently used to detect prions in cattle. At present, it is not possible to diagnose the disease in the pre-clinical phase or in live individuals using body biological fluids (24). The successful implementation of saPMCA should lead to the identification of individuals and tissue samples infected with even the tiniest quantities of PrP<sup>Sc</sup>, enabling us to decrease the risk of further propagation of TSE to the minimum. Indeed, we have recently reported for the first time the biochemical detection of PrP<sup>Sc</sup> in blood samples using saPMCA both at the symptomatic (16) and pre-symptomatic stages of the disease (35). Although technically more challenging, the PMCA technology has been adapted to amplify prions from a variety of origins, including human (14). One of the major limitations in the case of human samples is the availability of normal brain tissue to use as a substrate of PMCA, but this can be overcome by using transgenic mice brain or cell lysates.

Another important application of PMCA is to understand the underlying biology of prions and the nature of the infectious agent. In this study we show that it is possible to generate infectivity by amplification of a scrapie brain sample diluted 10<sup>-12</sup>-fold, containing an estimated single particle of oligomeric PrP<sup>Sc</sup>. By successive in vitro replication of this single particle of PrP<sup>Sc</sup>, we have successfully generated many millions of PrP<sup>Sc</sup>...
molecules and raised infectivity by more than 7 orders of magnitude. These data support and extend our previous findings showing the in vitro generation of prion-infectious material by cell-free replication of PrPSc using PMCA (15). These findings provide strong support for the prion hypothesis. However, we cannot rule out that other molecules present in the brain homogenate may also contribute to infectivity.

In addition, PMCA may be useful for understanding the molecular mechanism of prion replication and the identification of endogenous factors modulating PrPSc formation. Indeed, Supattapone and co-workers (36–39) have used PMCA to show that metal cations, such as copper and zinc, and polyamines, including diverse types of RNA molecules, can modulate PrP conversion in vitro. PMCA may also be used to examine and quantify the species barrier phenomenon and to understand the mechanism encoding prion strains. Finally, PMCA may be used as an efficient high throughput screening assay for identification of molecules able to inhibit or reverse prion replication and thus to discover novel potential drugs for TSE treatment. An efficient treatment to stop further prion conversion coupled with an early pre-symptomatic diagnosis to identify patients before irreversible brain damage has occurred seems the most promising approach to combat these devastating diseases.

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