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Prion formation, but not clearance, is supported by protein misfolding cyclic amplification

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Prion diseases are fatal transmissible neurodegenerative disorders that affect animals including humans. The kinetics of prion infectivity and PrP^{Sc} accumulation can differ between prion strains and within a single strain in different tissues. The net accumulation of PrP^{Sc} in animals is controlled by the relationship between the rate of PrP^{Sc} formation and clearance. Protein misfolding cyclic amplification (PMCA) is a powerful technique that faithfully recapitulates PrP^{Sc} formation and prion infectivity in a cell-free system. PMCA has been used as a surrogate for animal bioassay and can model species barriers, host range, strain co-factors and strain interference. In this study we investigated if degradation of PrP^{Sc} and/or prion infectivity occurs during PMCA. To accomplish this we performed PMCA under conditions that do not support PrP^{Sc} formation and did not observe either a reduction in PrP^{Sc} abundance or an extension of prion incubation period, compared to untreated control samples. These results indicate that prion clearance does not occur during PMCA. These data have significant implications for the interpretation of PMCA based experiments such as prion amplification rate, adaptation to new species and strain interference where production and clearance of prions can affect the outcome.

Introduction

Prion diseases are inevitably fatal neurodegenerative diseases affecting animals. The prion agent is likely solely comprised of a misfolded isoform of the prion protein, PrP^{Sc}, which is post-translationally derived from the host encoded normal isoform of the prion protein, PrP^{C}.[1,2,6] PrP^{Sc} can adopt a wide range of different conformations, which are thought to account for the variety of prion strains that can exist in a single PrP genotype in a given species.[10-12]

Formation of PrP^{Sc} is thought to occur in a 3-step process. First, PrP^{C} binds to PrP^{Sc} likely through the N-terminal and central polybasic domains.[15-16] Although other docking sites have been identified, they do not support formation of infectious prions.[18] Second, PrP^{Sc} directs the conversion of PrP^{C} to the PrP^{Sc} conformation by an unknown mechanism resulting in elongation of the PrP^{Sc} fibril. Finally, fragmentation of the PrP^{Sc} fibril results in the formation of new free ends for additional PrP^{C} binding to take place, thus completing the cycle. The rate of increase in prion infectivity and PrP^{Sc} accumulation can differ between strains and within a strain in different tissues.[20-22]

Fragmentation of PrP^{Sc} may contribute to the rate of prion formation. Initial studies of Sup35 fibrils, from the yeast prion [PSI+], have low conformational stability corresponding with high conversion rates.[24] Murine prion strains with short incubation periods correspond with PrP^{Sc} with low conformational stability compared to strains with longer incubation periods.[25,27] These data suggest that a decrease in conformational stability of the protein aggregate results in an increase in fragmentation that leads to an increase in prion formation. In both mammalian and yeast prions, however, other studies indicate that this relationship is not always true suggesting that additional factors are involved in fragmentation.[29-32]

The net accumulation of PrP^{Sc} is controlled by the relationship between the rate of PrP^{Sc} formation and degradation. Elimination of neuronal PrP^{C} expression during the course of prion infection results in clearance of PrP^{Sc} in these cells and subsequent reversal of a subset of prion-induced neuropathology.[44,46] Overexpression of PrP^{C} results in shortened incubation periods, however, due to the dual role of PrP^{C} in both PrP^{Sc} formation and neurodegeneration, the exact mechanism responsible for shortening of the incubation period is unclear.[1,3] The mechanism of PrP^{Sc} clearance is not fully understood, but may occur in lysosomes and can be accelerated by autophagy.[5-8]

Protein misfolding cyclic amplification (PMCA) is a powerful technique that faithfully recapitulates PrP^{Sc} formation and prion infectivity in a cell-free system. PMCA uses a repeated series of incubation periods and sonication that leads to formation of PrP^{Sc} and prion infectivity.[9,11,13,32] The PMCA incubation period is thought to allow for PrP^{C} binding to PrP^{Sc} and subsequent conversion of PrP^{C} to PrP^{Sc}. The sonication step is

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thought to shear the growing PrP^{Sc} fibril providing additional free ends for PrP^{C} to bind and convert to PrP^{Sc}.[14] This process of repeated elongation and fragmentation of PrP^{Sc} fibrils leads to exponential PrP^{Sc} production.[17]

Relatively little is known about PMCA mediated prion clearance. Several factors of the PMCA procedure have the potential to inactivate prions. Sonication produces cavitation microbubbles that can have extreme localized pressure and temperature that can denature protein and has the potential to reduce prion infectivity.[19] Additionally, endogenous proteases in brain homogenate can truncate and degrade PrP^{Sc} over time.[23] The effect of the combination of heat, protease activity and detergent that is present in the PMCA conversion buffer on prion degradation is unknown.

In this study we investigated if PrP^{Sc} and/or prion infectivity degradation occurred as a result of PMCA. To accomplish this we performed PMCA under conditions that do not support PrP^{Sc} formation. PrP^{Sc} degradation and an extension of prion incubation period were not observed under the PMCA conditions with the prion strains used. These data have significant implications on the interpretation of PMCA based experiments.

Results

PMCA formation of PrP^{Sc}

To confirm that amplification via PMCA is working within our standard parameters, either HY TME or DY TME-infected brain homogenate was diluted into uninfected hamster brain homogenate and subjected to one round of PMCA to serve as a positive control. Western blot analysis of PK treated samples demonstrated a significant ($P < 0.05$) increase in PK resistant PrP^{Sc} in the PMCA treated samples compared to the untreated controls and migration of the PMCA generated PrP^{Sc} maintained strain-specific patterns (**Fig. 1A and B**). Consistent with previous studies, HY PrP^{Sc} amplified to a significantly ($P < 0.05$) greater amount (**Fig. 1A**, lanes 5–6; Panel B) compared to DY PrP^{Sc} (**Fig. 1A**, lanes 9–10; Panel B). Formation of PrP^{Sc} was not observed in the mock seeded negative control reactions (**Fig. 1A**, lanes 11–12).

PMCA induced degradation of PrP is not observed

To investigate if conversion buffer and temperature induced PrP degradation, uninfected hamster brain homogenate alone and HY TME or DY TME-infected brain homogenates diluted in uninfected $MoPrP^{0/0}$ brain homogenate were incubated at 37°C for 24 hours without sonication. The abundance of PrP before (**Fig. 2A**, lanes 1–3) and after (**Fig. 2A**, lanes 4–6) the 37°C incubation did not differ significantly ($P > 0.05$) (**Fig. 2B**).

To investigate the effect of sonication on PrP degradation, HY TME or DY TME-infected brain homogenates were diluted in either DPBS (without conversion buffer components i.e. EDTA, Triton X-100 and protease inhibitors) or uninfected $MoPrP^{0/0}$ brain homogenate and subjected to one round of PMCA. The abundance of PrP^{Sc} in samples subjected to one round of PMCA or unsonicated samples in either $MoPrP^{0/0}$ brain (**Fig. 3A**, lanes 1–4) or DPBS (**Fig. 3A**, lanes 5–8) did not differ significantly ($P > 0.05$) (**Fig. 3B**). To study the effect of temperature and sonication on PrP^{C}, uninfected brain homogenate was subjected to one round of PMCA. The abundance of PrP^{C} did not differ significantly ($P < 0.05$) from the unsonicated control samples. (**Fig. 3C and D**). As a negative control, uninfected hamster brain homogenate diluted in $MoPrP^{0/0}$ brain homogenate was subjected to one round of PMCA. In these samples, Western blot analysis failed to detect PrP^{Sc} (data not shown).

Reduction of prion infectivity is not mediated by PMCA

To investigate the effect of PMCA on prion infectivity, HY TME-infected brain homogenate was diluted in $MoPrP^{0/0}$ brain homogenate and was subjected to either one round of PMCA, incubated at 37°C without sonication or left untreated as a positive control. These samples were i.c. inoculated into Syrian hamsters to determine if these treatments affected the incubation period of disease. All of the hamsters (n = 5) in each group developed clinical signs of hyperexcitability and ataxia. Animals inoculated with either the sonicated HY TME in $MoPrP^{0/0}$, HY TME in $MoPrP^{0/0}$ incubated at 37°C for 24 hours or the HY TME positive control had similar ($P > 0.05$) incubation periods of 72 ± 2, 71 ± 8 and 71 ± 3 d respectively (**Fig. 4A**). Western blot analysis of PK treated brain homogenates from clinically ill animals demonstrated the accumulation of PrP^{Sc}, confirming the clinical diagnosis (**Fig. 4B**, lanes 3–6). The

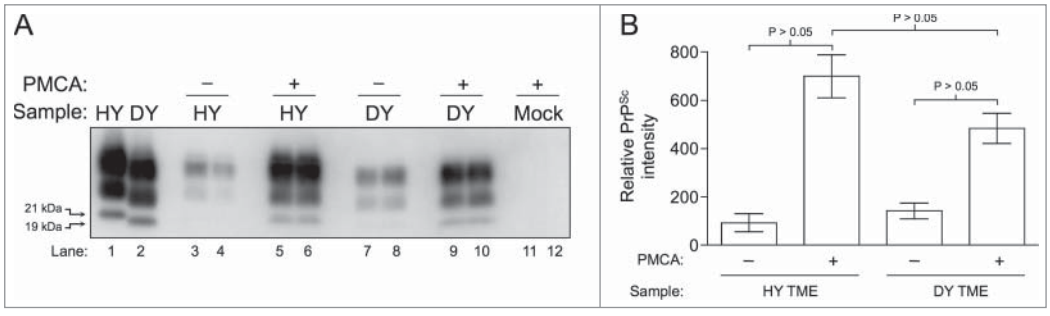

Figure 1. *In vitro* amplification of hamster adapted TME. (**A**) HY TME and DY TME were diluted in hamster brain homogenate and subjected to 144 cycles of 5-second sonication and 10 minute incubation. Following PK digestions, Western blot analysis show amplification of PrP^{Sc} when compared to their unsonicated controls (lanes 3–4 vs. 5-6 for HY TME and lanes 7-8 vs. 9-10 for DY TME). HY TME amplifies more efficiently than DY PrP^{Sc} (compare lanes 5-6 vs. 9-10). Mock: mock infected negative control (lanes 11–12). The migration of the 19 and 21 kDa unglycosylated PrP^{Sc} polypeptides is indicated on the left of panel A. (**B**) Bar graph comparing the relative PrP^{Sc} intensity before and after PMCA using HY TME or DY TME as a PrP^{Sc} seed (n = 5 per experimental group).

electrophoretic migration of PrP^{Sc} from either the sonicated HY TME or HY TME incubated at 37°C for 24 hours were similar to that of control HY TME PrP^{Sc} (**Fig. 4B**). None (n = 5) of the hamsters inoculated with sonicated mock-uninfected brain homogenate developed clinical signs of prion infection up to 360 d post infection, when the experiment was terminated (**Fig. 4A**). This was confirmed by Western blot of the PK digested brain tissue, which failed to detect the presence of PrP^{Sc} (**Fig. 4B**, lanes 7 and 8).

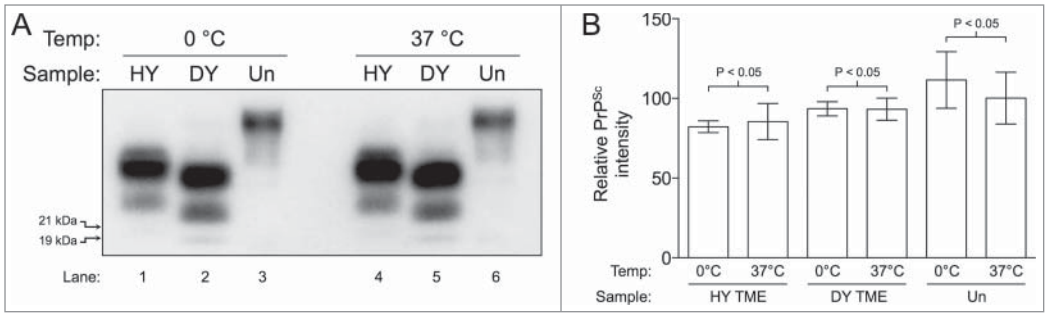

Figure 2. Temperature does not facilitate degradation of PrP. (**A**) HY TME and DY TME diluted in $MoPrP^{0/0}$ brain homogenate and uninfected hamster brain homogenate incubated at 0°C without sonication (Lanes 1–3) and at 37°C without sonication (Lanes 4–6). The migration of the 19 and 21 kDa unglycosylated PrP^{Sc} polypeptides is indicated on the left of panel A. (**B**) Bar graph comparing the relative intensities of the Western blot analysis of each sample before and after incubation (n = 3 per experimental group).

Discussion

PMCA can efficiently amplify large amounts of PrP^{Sc} and prion infectivity in an *in vitro* system.[11,26] Several aspects of prion biology have been accurately recapitulated using PMCA such as interspecies transmission, adaptation, replication co-factors, strain formation and environmental interactions.[18,26,28,30,32-42] While PMCA effectively recapitulates *in vivo* prion formation in many respects, little is known about the role of prion clearance during PMCA. Under conditions that do not support prion formation, we failed to find evidence of a reduction of PrP^{Sc} abundance or a change in incubation period due to the PMCA process. These observations are consistent with reports indicating that PrP^{Sc} can survive high levels of heat and proteolytic activity.[43,45,47] While not directly measured, it is likely that protease sensitive forms of PrP^{Sc} survive the PMCA process since the abundance of PrP^{C}, which is also sensitive to protease digestion, is unaltered. Overall, this data indicates that the net increase in PrP^{Sc} and infectivity in PMCA is due solely to PrP^{Sc} formation, and is not the result of a dynamic balance between formation and clearance in contrast to what occurs *in vivo*. This observation affects the interpretation of PMCA based experiments and might contribute to the ability of PMCA to amplify PrP^{Sc} amounts that are below the limit of detection in bioassay.

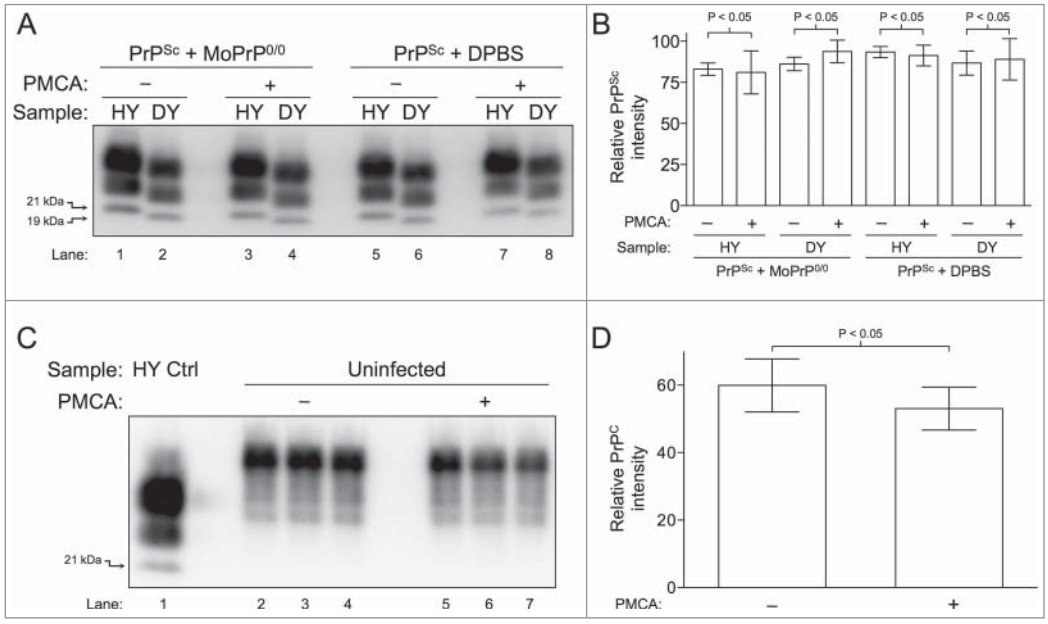

Figure 3. Clearance of PrP is not supported by PMCA. (**A**) Western blot of HY TME and DY TME showing an absence of PrP^{Sc} clearance during PMCA. HY TME and DY TME diluted in $MoPrP^{0/0}$ brain homogenate without sonication (Lanes 1–2); HY TME and DY TME diluted in $MoPrP^{0/0}$ brain homogenate subjected to PMCA (Lanes 3–4); HY TME and DY TME diluted in DPBS without sonication (Lanes 5–6); and HY TME and DY TME diluted in DPBS subjected to PMCA (Lanes 7–8). (**B**) Bar graph comparing the relative intensity of each sample before and after sonication (n = 4 per experimental group). (**C**) Western blot of uninfected hamster brain homogenate showing the absence of PrP^{C} clearance during PMCA. Uninfected brain homogenate without PMCA (Lanes 2–4) and after PMCA (Lanes 5–7). (**D**) The relative average intensities of PrP^{C} as quantified from the Western blot analysis of each sample before and after PMCA (n = 3 per experimental group). The migration of the 19 or 21 kDa unglycosylated PrP^{Sc} polypeptides is indicated on the left of panels A and C.

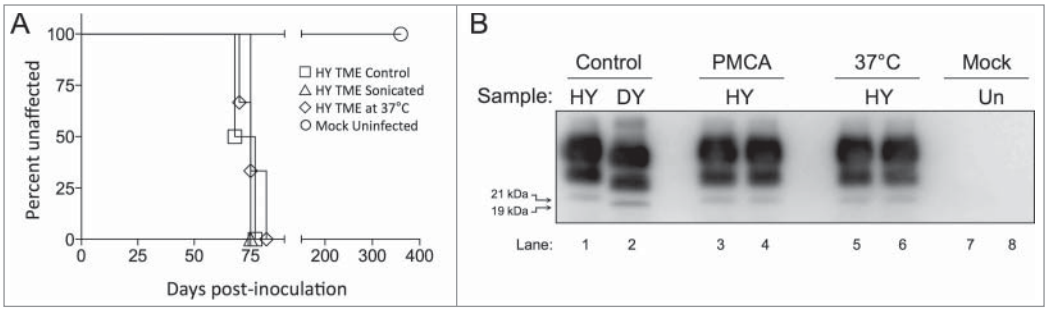

Figure 4. PMCA treatment of HY TME does not affect its infectivity. (**A**) Survival of Syrian hamsters following i.c. inoculation of HY TME control (□) HY TME in MoPrP$^{0/0}$ subjected to one round of PMCA (△) HY TME in MoPrP$^{0/0}$ at 37°C without sonication (◇) and Mock inoculated hamster (○) (n = 5 per experimental group). (**B**) Western blot analysis of brain homogenate from hamsters inoculated with sonicated HY TME in MoPrP$^{0/0}$ (lanes 3–4); HY TME in MoPrP$^{0/0}$ incubated at 37°C without sonication (lanes 5–6); and mock uninfected (lanes 7–8). The migration of the 19 and 21 kDa unglycosylated PrP^{Sc} polypeptides is indicated on the left of panel B.

Accurate measurements of prion formation *in vivo* have not been made. In animals both prion formation and clearance contribute to the net accumulation of PrP^{Sc} and infectivity.[48-50] Studies utilizing PMCA have been used to calculate the efficiency of PrP^{Sc} formation, however, the possibility that prion clearance contributed to the net accumulation of PrP^{Sc} and infectivity could not be excluded.[13,32,38,40] The results of this study indicate that PMCA provides an accurate measurement of prion formation. Strain specific differences in PMCA PrP^{Sc} formation efficiency have been observed and must be due to strain-specific differences in prion formation and not clearance.[32]

Adaptation of prions to a new host species using PMCA can effectively overcome the species barrier. This process can also result in the identification of novel prion strains when transmitted to the new host species.[33,51-54] One possibility for this observation is that PMCA allows for the formation of PrP^{Sc} conformations (i.e., sub strains) that are not favored *in vivo*. Alternatively, identical populations of PrP^{Sc} conformations are produced both *in vivo* and in PMCA, but a subpopulation of PrP^{Sc} conformations are cleared *in vivo* that are not cleared in PMCA. This could result in the evolution of PrP^{Sc} conformations that gain the ability to survive *in vivo* clearance mechanisms or can alter the balance of prion strains in a mixture, which has been shown to influence strain emergence consistent with the prion quasispecies hypothesis.[2,4,55-61]

Prion strains in a mixture do not act independently but rather interfere with each other.[6,62] The mechanism(s) for strain interference are only beginning to be understood.[10,12,30,63] One unexplored possibility is that infection with one prion strain can facilitate the clearance of a second strain and contribute to strain interference. This has not been possible to investigate *in vivo*. Strain interference can occur using PMCA and the same experimental parameters that influence strain interference *in vivo* apply to PMCA strain interference.[15,16,30] Based on the observation of a lack of clearance of PrP^{Sc} in PMCA in combination with the ability of PMCA to accurately model strain interference *in vivo*, we hypothesize that prion clearance is not involved in strain interference. Overall, the inability of PMCA to support prion clearance is an important variable to fully interpret PMCA based studies and can be exploited to explore the relationship between prion formation and clearance.

Materials and Methods

Prion strains and negative controls

Brains from terminally-ill hamsters inoculated with either biologically cloned HY or DY TME agents were homogenized to 10% w/v in Dulbecco's phosphate buffered saline (DPBS) (Mediatech, Herndon, VA). Uninfected hamster brain or PrP^{C} knock-out mice brain MoPrP$^{0/0}$ was homogenized to 10% w/v in PMCA conversion buffer (phosphate buffer saline containing 1% v/v Triton-X100, 5 mM EDTA with protease inhibitors).[64] All homogenates were stored at −80°C prior to use.

Protein misfolding cyclic amplification

PMCA was performed as described previously.[65] Briefly, we used a Misonix 3000 sonicator (Farmingdale, NY) coupled to a 96-well plate titanium cup-horn. The sonicator output was set to level 6. With the sonicator cup horn filled with distilled water at 37°C the average power output was 160 W. One round of PMCA consisted of 144 cycles of 5 seconds sonication and 10 minutes incubation per cycle. The PMCA reactions contain 20 μl of 10% w/v brain homogenates from either HY TME or DY TME-infected hamsters diluted into 80 μl of 10% w/v uninfected hamster or mouse PrP$^{0/0}$ brain homogenate in PMCA conversion buffer or DPBS and were subjected to one round of PMCA. The negative control PMCA reaction consisted of uninfected hamster brain homogenate. All PMCA reactions were replicated a minimum of 3 times.

SDS-PAGE and Western blot analysis

The PMCA reactions were digested with 0.4 U/ml of proteinase K (PK) at 37°C for 30 minutes with constant agitation (Roche Diagnostics Corporation, Indianapolis, IN). The PK digestion was terminated by incubating the samples at 100°C for 10 minutes in gel loading buffer (4% SDS, 2% β-mercapto ethanol, 40% glycerol, 0.004% Bromophenol blue, and 0.5 M Tris buffer pH 6.8). SDS-PAGE and Western blot analysis were performed as described previously using the anti-PrP antibody 3F4 (final concentration of 0.1 μg/ml; Chemicon; Billerica, MA) to recognize hamster prion protein.[30] The Western blot was developed with Pierce supersignal west femto maximum sensitivity substrate according to manufacturer instructions (Pierce, Rockford, IL) and imaged on a Kodak 4000R Imaging Station (Kodak, Rochester, NY).

The abundance of PK resistant PrP^{Sc} was determined using the Kodak molecular imaging software v.5.0.1.27 (New Haven, CT) as described previously.[30]

Animal bioassay

All procedures involving animals were approved by the Creighton University Institutional Animal Care and Use Committee and were in compliance with the Guide for the Care and Use of Laboratory Animals. Intracerebral inoculations were performed on 3–4 week old male Syrian hamsters (Harlan-Sprague-Dawley, Indianapolis, IN) as previously described.[66] Groups of 5 hamsters were intracerebrally inoculated with 25 μl of the PMCA treated and unsonicated samples diluted to 1% w/v in DPBS. Hamsters were observed 3 times per week for the onset of clinical signs of prion disease and the incubation period was calculated as the number of days between inoculation and onset of clinical signs.[67]

Statistical analysis

Student's t test analysis was performed using Prism 6 for Mac (GraphPad Software Inc., La Jolla, CA).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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