

Protein Misfolding Cyclic Amplification Cross-Species Products of Mouse-Adapted Scrapie Strain 139A and Hamster-Adapted Scrapie Strain 263K with Brain and Muscle Tissues of Opposite Animals Generate Infectious Prions

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Abstract Transmission of prions between mammalian species is limited by a “species barrier,” a biological effect involving an increase in incubation period to decrease the percentage of animals succumbing to disease. In this study, we used protein misfolding cyclic amplification (PMCA) technique, which accelerates the conversion of prion proteins *in vitro*. Direct interspecies PMCA involving 144 cycles confirmed that both mouse-adapted scrapie strain 139A and hamster-adapted 263K could use brain homogenates of opposite species to form proteinase K (PK)-resistant PrP proteins (PrP^{res}). Newly formed interspecies prions could stably propagate themselves in subsequent serial PMCA passages. The two types of PMCA-generated cross-species PrP^{res} changed their glycosylation profiles, which was similar to that observed during interspecies infection by the mouse agent 139A *in vivo*. These profiles were distinct from individual seeded PrP^{Sc} and possessed properties of new hosts. Comparative

analysis with respect to PK resistance showed no significant diversity between PMCA-PrP^{res} and native PrP^{Sc} or between brain and muscle PrP^{res}. However, PrP^{res} from the relatively early cycles of serial PMCA showed lower PK resistance than those from later cycles. Inoculation of these PMCA products amplified with homogeneous or heterogeneous brain tissues (cross-species products) induced experimental transmissible spongiform encephalopathies. These results suggested that PMCA can help prion strains to overcome species barrier and to propagate efficiently both *in vitro* and *in vivo*.

Keywords Prion · Strain · Species barrier · Protein misfolding cyclic amplification (PMCA) · Glycosylation · Transmissible spongiform encephalopathies (TSEs)

Introduction

Prion diseases (also known as transmissible spongiform encephalopathies [TSEs]) affect the brain of different animals having similar neuropathological features. TSEs include Creutzfeldt–Jakob disease (CJD) and Kuru in humans, bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep, and chronic wasting disease in cervids [1]. TSEs are caused by unconventional infectious agents called prions, which lack nucleic acids. A host-encoded glycoprotein called cellular prion protein (PrP^C) changes into a misfolded conformer PrP^{Sc}, which accumulates mainly in the central nervous system [2]. A classical triad of spongiform change, neuronal loss, and gliosis (astroglia and microglia) is the neuropathological hallmark of prion diseases.

TSEs share two characteristics with conventional infectious agents [1]. One characteristic is the presence of multiple prion strains that after inoculation in the same host induce

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specific and stable phenotypic traits such as incubation period, molecular pattern of PrP^{Sc}, and neuropathology [3]. Protein-only hypothesis proposes that these strain-specific traits are induced by biologically active, structural differences among PrP^{Sc} molecules [2]. The other characteristic is species barrier, a transmission barrier that restricts the propagation of prions between different species. Its biological effect is reflected by the longer incubation time or the complete resistance in animals inoculated with other strains of TSE species.

Cross-species transmission of a TSE agent was first demonstrated using a sheep scrapie agent in laboratory rodents in the 1930s. The most well-known example is the outbreak of human variant CJD (vCJD) in 1996 in the UK and France, which is closely associated with the BSE endemic in Europe. Many studies have investigated mechanisms underlying the transmission of TSE agents and have proposed some biological factors that contribute to the efficiency of cross-species transmission of TSE. New findings indicate that sialylation of prion proteins controls the rate of prion amplification and cross-species transmission [4]. Diversity of the host gene encoding the PrP protein (*PRNP*) also seems to be an essential factor. Although *PRNP* is conserved among species during evolution and the tri-dimensional structure of PrP^C is highly conserved among mammalian species, a few amino acid divergences among species can greatly affect the transmission efficiency.

Prion strains also play a pivotal role in the cross-species transmission of TSEs. Bank voles can be easily infected experimentally by using prion agents from the brains of animals with sporadic and genetic CJD despite a relatively large difference in *PRNP* genes between humans and voles [5]. *PRNP* genes of mice and hamsters are similar. However, a mouse-adapted scrapie agent can be experimentally transmitted to hamsters relatively easily but a hamster-adapted scrapie agent induces TSE in mice with higher difficulty [5]. Therefore, most species barriers can be perceived as strain barriers [6]. Route of infection is another critical factor that modifies the magnitude of transmission barrier. In some hosts, PrP^C sequence affects prion distribution within the lymphoid tissue [7]. Propagation of PrP^{Sc} in the extra neural tissue is also important for the efficacy of cross-species transmission [8].

Protein misfolding cyclic amplification (PMCA) is a technique that accelerates the conversion of PrP^C into PrP^{Sc} in vitro. PMCA has been adapted for many different applications, including PrP^{Sc} detection in body fluids and tissues, screening for inhibitors against prion replication and demonstrating the intriguing phenomena of prion strains and species barriers [9]. Detection of prion replication or prion diseases usually takes a long time in vivo. The ability of PMCA to accelerate prion propagation in vitro may help in preventing the death of experimental animals before the appearance of the disease or before the detectable accumulation of PrP^{Sc} in their brains. Previously, we successfully transmitted mouse-adapted scrapie strains 139A and ME7 to hamsters by prolonging their

incubation period [10]; however, we failed to infect C57BL/6 mice with hamster-adapted scrapie agent 263K. In this study, mouse agent 139A and hamster agent 263K were successfully amplified in vitro by performing PMCA with brain and muscle homogenates of opposite animals. The new PMCA-generated cross-species prions were inoculated into hamsters or mice to induce typical experimental TSEs. Our data indicated that both hamster-adapted scrapie agent 263K and mouse-adapted scrapie agent 139A easily crossed the species barrier after PMCA and induced a typical prion infection in the opposite animals.

Results

Scrapie Mouse-Adapted Strain 139A and Hamster-Adapted Strain 263K Can Interspecies Replicate in PMCA with Opposite Brain and Muscle Homogenates

Our previous study showed that mouse-adapted scrapie strain 139A induced experimental scrapie in hamsters after a prolonged incubation period [10]. To determine whether strain 139A from mouse brains efficiently replicated in vitro with PrP^C from hamsters as substrate, 10 % brain and muscle homogenates of normal hamsters were mixed with different concentrations of 139A-infected mouse brain homogenates (10²- to 10⁵-fold dilution). A mixture of 10 % brain homogenate of normal mouse and brain homogenate of 139A-infected mouse was used as a positive control (Seed Ctrl), and brain or muscle homogenate of hamster as substrate alone was used as a negative control (Substrate Ctrl). Serially diluted 139A seeds (10²- to 10⁵-fold dilution) in PMCA buffer without any substrate were used as buffer control. These preparations were subjected to 144 cycles of direct PMCA. PMCA products were treated with 50 µg/ml PK and were immunoblotted using PrP-specific mAb 3F4, which recognizes hamster-derived PrP, or 6D11, which recognizes both hamster- and mouse-derived PrPs. PK-resistant PrP (PrP^{res}) bands were observed in the positive control blotted with mAb 6D11 (Fig. 1a) but not in the positive control blotted with mAb 3F4 (Fig. 1b). PrP^{res} signal was detectable in the buffer control containing 10⁻² dilution of scrapie strain 139A (Fig. 1c) but not in the substrate control (Fig. 1d). As expected, clear PrP^{res} signals were observed in PMCA products of scrapie strain 139A (10⁻²–10⁻⁴ dilution) mixed with hamster brain or muscle homogenates substrates after blotting with mAbs 6D11 (Fig. 1a) and 3F4 (Fig. 1b). These data indicated that PMCA caused the mouse-derived prion strain 139A to induce the conversion of hamster-derived PrP^C to PrP^{res}. The newly formed PrP^{res} molecules seemed to be hamster derived because they were recognized by both mAbs 6D11 and 3F4.

To assess whether the hamster-adapted scrapie strain induced the conversion of mouse PrP^C to PrP^{res}, 10⁻²–10⁻⁵

Seed: 139A-mouse

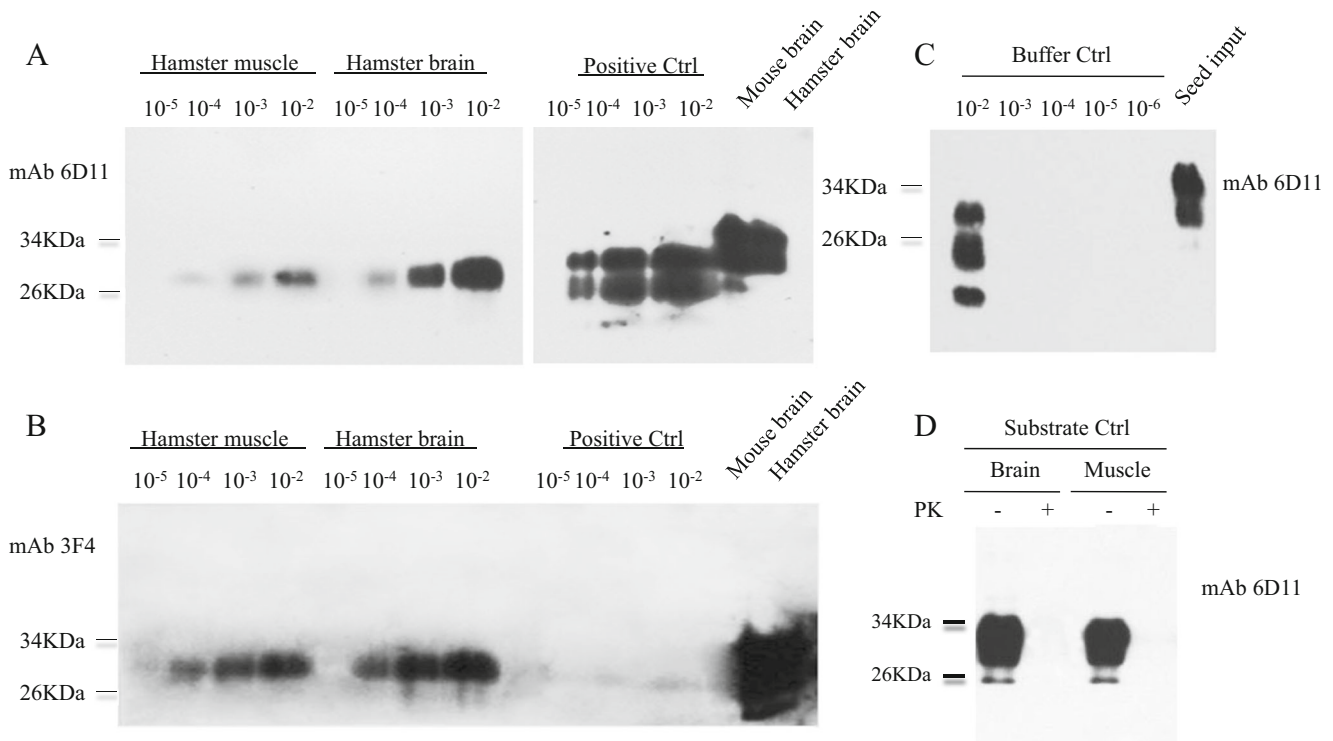


Fig. 1 In vitro conversion of hamster PrP^C induced by mouse-adapted scrapie strain 139A. Strain 139A-infected brain homogenate was diluted by 10²–10⁵-fold by using normal hamster brain or muscle homogenates and was subjected to 144 cycles of direct PMCA. All the products were subjected to PK digestion (final concentration, 50 μg/ml) at 37 °C for 1 h. **a** Western blotting of PMCA products with mAb 6D11 by using hamster tissue homogenates as the substrate. **b** Western blotting of PMCA products with mAb 3F4 by using hamster tissue homogenates as the

substrate. Substrates and dilution folds used in the reaction are shown on the top. Mouse and hamster brains represent 10 % brain homogenates loaded directly as control on the right side. **c** Agent 139A was serially diluted in conversion buffer and was used as a buffer control in direct PMCA. The reactions were blotted using mAb 6D11. **d** Next, 10 % hamster brain and muscle homogenates without 139A seeds were subjected to direct PMCA as substrate controls. The reactions were blotted using mAb 6D11. Molecular weight standards are shown on the left

dilutions of 263K-infected hamster brain homogenates were mixed with 10 % brain or muscle homogenates of normal mouse and were subjected to 144 cycles of direct PMCA. Western blotting of PMCA products with both mAbs 3F4 (Fig. 2a) and 6D11 (Fig. 2b) showed obvious PrP^{res} signals in the positive control of 263K seed (10⁻²–10⁻⁴ dilution) with hamster brain PrP^C substrates. PrP^{res} signals were detectable in the buffer control containing 10⁻² dilutions of agent 263K and were weakly detectable in the buffer control containing 10⁻³ dilutions of agent 263K after blotting with mAb 3F4 (Fig. 2c). No PrP^{res} signal was detectable in the substrate control of hamster brain or muscle homogenates (Fig. 2d). Interestingly, clear PrP^{res} signals were observed in PMCA products of agent 263K mixed with both mouse brain and muscle homogenates blotted with mAb 6D11 (Fig. 2b) but not in those blotted with mAb 3F4 (Fig. 2a). This indicated that hamster-adapted scrapie strain 263K could convert normal mouse PrP^C to PrP^{res} during PMCA and that the newly generated PrP^{res} possessed the characteristics of mouse PrP.

To investigate the possible differences in the efficacy of replication of the two scrapie agents with mixtures of infected

and normal brain homogenates in vitro, the tissues were subjected to 48, 96, and 144 cycles of PMCA. After 48 cycles with individual homologous brain homogenates as substrates, both mouse-adapted agent 139A and hamster-adapted agent 263K produced PK-resistant PrP (PrP^{res}) signals after blotting with mAb 6D11 (data not shown). However, in mixtures containing heterologous brain homogenates, both the agents showed obvious replication in experiments involving 144 cycles but not in experiments involving 48 and 96 cycles. Under our experimental conditions, no differences were observed in the efficacy of replication of the 2 scrapie agents by using brain tissues of the opposite animals as substrates.

The Two Types of Newly Formed PrP^{Sc} Can Be Propagated In Vitro by Serial PMCA

To examine whether the newly generated cross-species PrP^{res} could be propagated continually in vitro, various PMCA products were subjected to serial PMCA with individual normal tissue homogenates as substrates. Briefly, 10 μl PMCA products was diluted 10-fold with 90 μl of 10 % normal tissue homogenate and

Seed: 263K-hamster

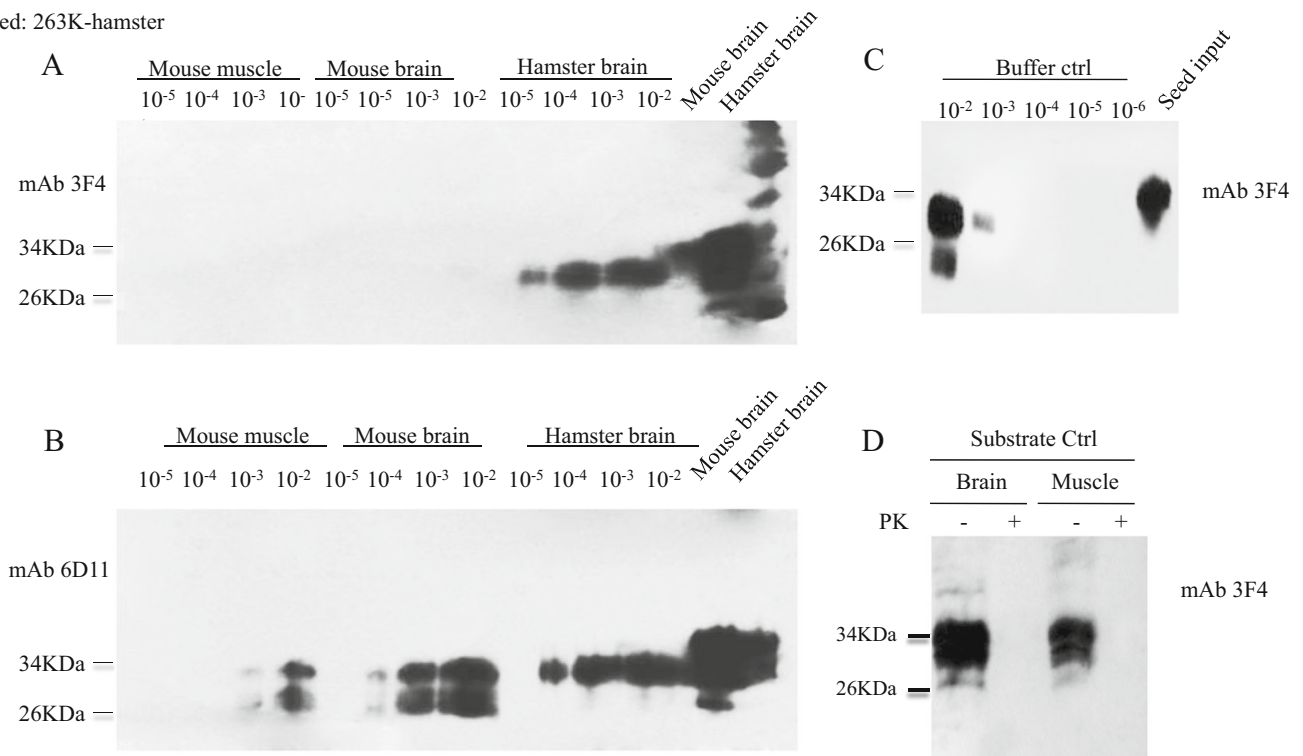


Fig. 2 In vitro conversion of mouse PrP^C induced by hamster-adapted scrapie strain 263K. Strain 263K-infected brain homogenate was diluted by 10^2 – 10^5 -fold by using normal mouse brain or muscle homogenates and was subjected to 144 cycles of PMCA. All the products were subjected to PK digestion (final concentration, 50 μ g/ml) at 37 °C for 1 h. **a** Western blotting of PMCA products with mAb 3F4 by using mouse tissue homogenates as the substrate. **b** Western blotting of PMCA products with mAb 6D11 by using mouse tissue homogenates as the

substrate. The substrates and dilution folds used in the reaction are shown on the top. Mouse and hamster brains represent 10 % brain homogenates loaded directly as control on the right side. **c** Agent 263K was serially diluted in conversion buffer and was used as a buffer control in direct PMCA. The reactions were blotted using mAb 3F4. **d** Next, 10 % mouse brain and muscle homogenates without 263K seeds were subjected to direct PMCA as substrate controls. The reactions were blotted using mAb 3F4. Molecular weight standards are shown on the left

was subjected to one round of PMCA with 144 cycles. The same process was repeated for the next round of PMCA until the final concentration of the original input PrP^{Sc} reached 10^{-17} dilution. An aliquot of the PMCA product was used for further assays after completing one round of PMCA. Western blotting with mAb 6D11 showed stable PrP^{res} signals in all preparations containing 10^{-9} – 10^{-17} dilutions of the scrapie agents, including 139A hamster-generated PrP^{res} with brain and muscle homogenates (Fig. 3a, b) and 263K mouse-generated PrP^{res} with brain and muscle homogenates (Fig. 3c, d). No PrP^{res} signal was observed in individual substrate controls, including brain and muscle homogenates of hamsters and mice, after serial PMCA (Supplemental Fig. 1). This implied that the newly formed cross-species PrP^{res} used PrP^C from the new hosts to continuously replicate during PMCA.

Newly Formed PrP^{Sc} Change Their Glycosylation Patterns During Interspecies Replication in PMCA

Mouse-adapted scrapie agent 139A and hamster-adapted scrapie agent 263K show different glycosylation profiles in the brains of infected animals, with monoglycosylated PrP^{Sc}

predominantly present in the brains of 139A-infected mice and diglycosylated PrP^{Sc} predominantly present in the brains of 263K-infected hamsters. Our previous study showed that the PrP^{Sc} glycosylation profile of mouse agent 139A predominantly changed to the diglycosylated form after it stably infected hamsters [10]. Analyses of PrP^{res} glycosylation patterns during direct PMCA (Figs. 1 and 2) showed that mouse agent 139A-derived PrP^{res} with hamster tissue homogenates (139A-hamster PrP^{res}) formed a single band that mobilized toward the position of diglycosylated PrP^{res} and that hamster agent 263K-derived PrP^{res} with mouse tissue homogenates (263K-mouse PrP^{res}) formed multiple bands, with monoglycosylated PrP^{res} being the predominant form. These PrP^{res} glycosylating patterns were the same when both brain or muscle homogenates were used as substrates. Further, serial PMCA tests (Fig. 3) confirmed that these profiles were maintained stably during subsequent PMCA passages. Quantitative assay of the ratios of PrP^{res} signals showed that 263K-mouse PrP^{res} contained 33–35, 45–51, and 15–18 % di-, mono-, and unglycosylated PrP^{res}, respectively. In contrast, 139A-hamster PrP^{res} mostly contained diglycosylated PrP^{res} (Fig. 4). Further analysis of the glycosylation profiles of the two types

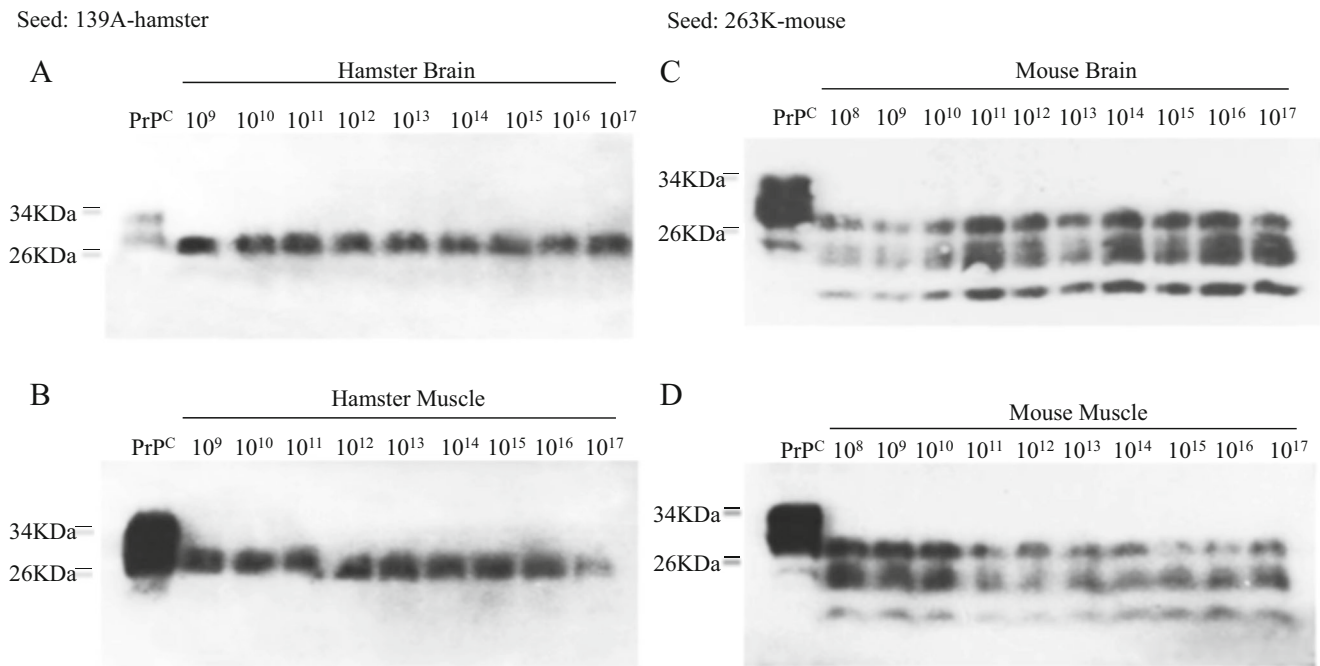


Fig. 3 Serial PMCA of mouse strain 139A and hamster strain 263K in the brain and muscle homogenates of opposite species. For this, 10 % infected rodent brain homogenates were mixed with 10-fold 10 % tissue homogenates of opposite normal animals and were subjected to 144 cycles of PMCA. PMCA products were mixed with 10-fold individual normal tissue homogenates and were subjected to the next round of PMCA until the final dilution of the original scrapie agents reached 10^{-17} . PMCA products in

reactions containing 10^9 - to 10^{17} -fold diluted original agents were blotted with mAb6D11 after PK digestion. **a** Agent 139A in normal hamster brain homogenates. **b** Agent 139A in normal hamster muscle homogenates. **c** Agent 263K in normal mouse brain homogenates. **d** Agent 263K in normal mouse muscle homogenates. Dilutions of original agents are shown on the *top*. Molecular weight standards are indicated on the *left*. Hamster or mouse brain homogenates were directly used as control (PrP^{C})

of interspecies PMCA PrP^{res} showed apparent host-associated features. 139A-hamster brain and muscle PrP^{res} had similar glycosylation profiles as 263K-hamster PrP^{Sc} (in infected brains) and 263K-hamster brain PrP^{res} (in the PMCA of PrP^{C} substrates from hamster brains). 263K-mouse brain and muscle PrP^{res} had similar glycosylation profiles as 139A-mouse PrP^{Sc} (in infected brains) and 139A-mouse brain PrP^{res} (in the PMCA of PrP^{C} substrates from mouse brains). These data suggested that the original scrapie seeds changed their glycosylation features during interspecies propagation in PMCA. These alterations may occur at the beginning of interspecies propagation during PMCA (i.e., the first round of serial PMCA) and may be maintained stably in further PMCA passages.

Newly Formed PrP^{res} Possess Similar and Stable PK Resistance as Their Parent Proteins

To evaluate the PK resistance of newly formed PrP^{res} , various PMCA products were digested using different concentrations of PK (50–1000 $\mu\text{g/ml}$). Brain homogenates of 139A-infected mice and 263K-infected hamsters were used as controls. PMCA products from rounds 6 to 8 were pooled as early PMCA products and those from rounds 13 to 17 were pooled as late PMCA products. Input PrP concentrations in various

samples were equilibrated by performing PrP-specific western blotting. PK-resistant PrP signals were detectable in the brain homogenates of both 139A-infected mice and 263K-infected hamsters, with a slighted crease in signal intensity in preparations containing 1000 $\mu\text{g/ml}$ PK. Similar to the above results, clear PrP^{res} signals were observed in all the preparations of PMCA products, irrespective of whether they were mouse- or hamster-derived products, brain- or muscle-derived products, and early or late PMCA products (Fig. 5). PrP^{res} signals remained stable after treatment with 50–500 $\mu\text{g/ml}$ PK and became weaker after treatment with 1000 $\mu\text{g/ml}$ PK. Results of quantitative assays of PrP^{res} signal intensity for each reaction were normalized with those of quantitative assay of 50 $\mu\text{g/ml}$ PK and showed comparable signal gray values for preparations containing 50, 100, and 500 $\mu\text{g/ml}$ PK. However, PrP^{res} signal intensity decreased in all the preparations containing 1000 $\mu\text{g/ml}$ PK (Fig. 5). Moreover, the early cycles (EC) of serial PMCA showed higher decrease PrP^{res} signal intensity than the late cycles (LC), with approximately 50–60 and 70–80 % decrease in signal intensity, respectively, for 50 $\mu\text{g/ml}$ PK (Fig. 5). These results indicated that the PK resistance capacity of PMCA-generated cross-species PrP^{res} was comparable to that of their original seed prions. PK resistance of the newly formed PrP^{res} may have been stabilized during PMCA passages.

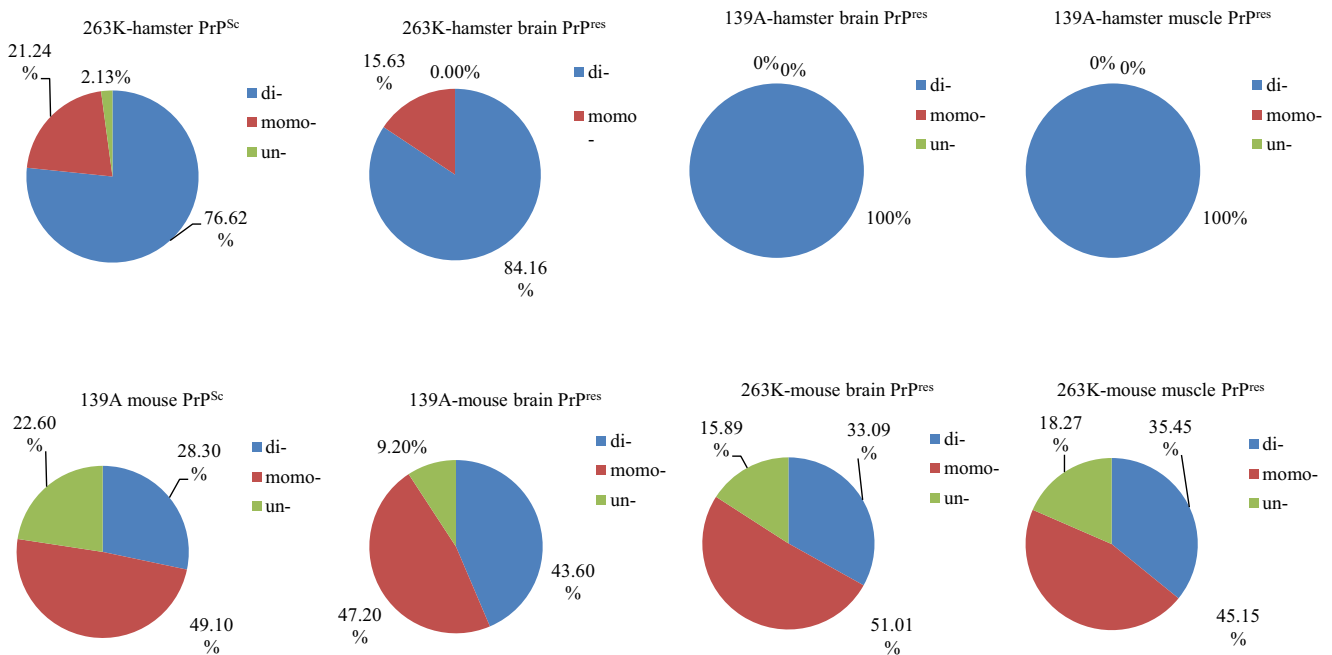
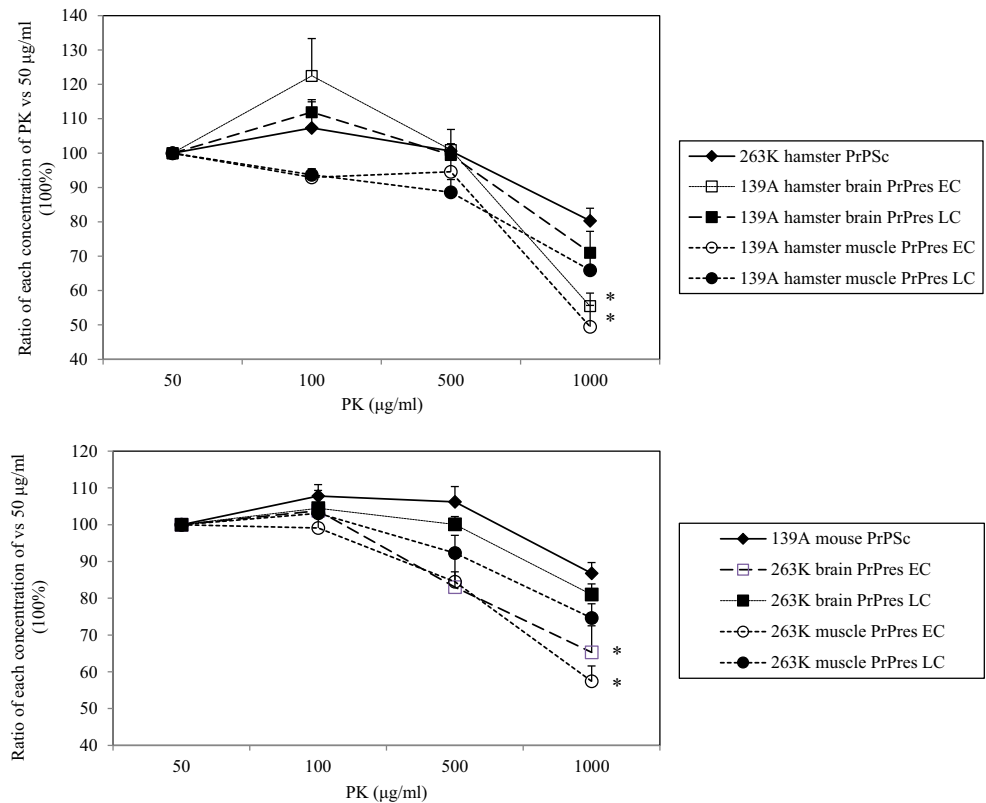


Fig. 4 Glycosylation patterns of newly formed PrP^{res} in PMCA and their parent PrP^{Sc} in infected brains. PrP^{res} signals were captured from the western blots of PMCA products. Average percentages of di-, mono-, and unglycosylated PrP^{res} in total PrP^{res} are shown. Individual PrP^{res} or PrP^{Sc} are indicated on the top. 263K-hamster PrP^{Sc}: PrP^{Sc} in the brains of 263K-infected hamsters; 263K-hamster brain PrP^{res}: PrP^{res} in the PMCA of 263K seed and hamster brain homogenates; 139A-hamster brain PrP^{res}:

PrP^{res} in the PMCA of 139A seed and hamster brain substrates; 139A-hamster muscle PrP^{res}: PrP^{res} in the PMCA of 139A seed and hamster muscle substrates. 139A-mouse PrP^{Sc}: PrP^{Sc} in the brains of 139A-infected mice; 139A-mouse brain PrP^{res}: PrP^{res} in the PMCA of 139A seed and mouse brain substrates; 263K-mouse brain PrP^{res}: PrP^{res} in the PMCA of 263K seed and mouse brain substrates; and 263K-mouse muscle PrP^{res}: PrP^{res} in the PMCA of 263K seed and mouse muscle substrates

Fig. 5 Quantitative analysis of PrP^{res} or PrP^{Sc} derived from hamster and mouse tissues after digesting with various concentrations of PK. Amplified products from rounds 6 to 8 were pooled as samples of the early cycle and those from rounds 13 to 17 were pooled as samples of the late cycle. Brain homogenates of 263K-infected hamsters and 139A-infected mice were used as parent PrP^{Sc}. Samples were mixed with different concentrations of PK (50, 100, 500, and 1000 µg/ml) and were subjected to western blotting with mAb 6D11. Average values are calculated from three independent tests, are normalized with individual data of reactions involving 50 µg/ml PK and are presented as mean (percentage) ± SD. *EC* early cycle, *LC* late cycle. **P* < 0.05



Inoculation of PMCA Cross-Species Products 139A-Hamster and 263K-Hamster into Hamster Induced Experimental TSEs

To assess the infectivity of PMCA-generated PrP^{res} in experimental rodents, various serial PMCA products were prepared using 139A-infected mouse brain and 263K-infected hamster brain homogenates as seeds and homologous and heterologous brain homogenates as substrates. For this, 10⁻¹⁷-fold dilution of input PrP^{Sc}, including 139A-hamster PMCA-PrP^{res}, 139A-mouse PMCA-PrP^{res}, 263K-hamster PMCA-PrP^{res}, and 263K-mouse PMCA-PrP^{res}, was used as the final concentration. Six hamsters were intracerebrally inoculated with an aliquot of 139A-hamster PMCA-PrP^{res}, 139A-mouse PMCA-PrP^{res}, or 263K-hamster PMCA-PrP^{res} separately. Further, six hamsters inoculated with brain homogenates of 263K-infected hamsters were used as positive controls. Similarly, six mice were intracerebrally inoculated with an aliquot of 263K-mouse PMCA-PrP^{res}, 263K-hamster PMCA-PrP^{res}, or 139A-mouse PMCA-PrP^{res} separately. Further, six mice inoculated with brain homogenates of 139A-infected mice were used as positive controls. Two hamsters in the 139A-hamster PMCA-PrP^{res}, 263K-hamster PMCA-PrP^{res}, and 263K-brain control groups and two mice in the 263K-mouse PMCA-PrP^{res} and 139A-mouse PMCA-PrP^{res} groups died within the first week after inoculation because of acute mechanical brain damage during inoculation. Information on bioassays is summarized in Supplemental Fig. 2.

As expected, hamsters inoculated with 263K-infected hamster brain homogenates showed clinical symptoms from 65 to 77 days post-inoculation (dpi) (Table 1 and Fig. 6a). Hamsters inoculated with 263K-hamster PMCA-PrP^{res} induced clinical symptoms similar to those in the positive control group between 40 and 71 dpi, with an average incubation period of 56 ± 14.4 days. Animals infected with 139A-hamster PMCA-PrP^{res} showed abnormal signs between 131 and 180 dpi, with an average incubation period of 157 ± 17.6 days. All hamsters inoculated with 139A-mouse PMCA-PrP^{res} did not show any abnormality until the end of the observation period (300 dpi).

Clinical manifestations in diseased animals were similar at the terminal stage. Severe loss of weight, extreme emaciation, and sluggishness were observed in all the diseased animals. Obvious ataxia was observed in approximately 50 % diseased hamsters. Clinical manifestations progressed quickly after onset. Average clinical courses of groups 139A-hamster PMCA-PrP^{res}, 263K-hamster PMCA-PrP^{res}, and positive control (brain tissues of 263K-infected hamsters) were 10.3 ± 3.1, 7.3 ± 3.5, and 7.0 ± 2.0 days, respectively. PrP-specific western blotting showed PK-resistant bands in all hamsters with clinical manifestations and no bands in hamsters infected with 139A-mouse PMCA-PrP^{res} that did not show any clinical symptoms (Table 1 and Fig. 6). Bands corresponding to diglycosylated PrP^{res} were significantly predominant in all the diseased animals. Glycosylation patterns determined by performing a quantitative assay of the ratios of PrP^{res} signals showed that the percentages of di-, mono-, and unglycosylated PrP^{res} were 45, 37, and 18 %, respectively, in animals infected with 139A-hamster PMCA-PrP^{res}; 63, 37, and 0 %, respectively, in animals infected with 263K-hamster PMCA-PrP^{res}; and 69, 31, and 0 %, respectively, in animals in the 263K-positive control group.

Positive control mice infected with agent 139A showed visible symptoms between 165 and 177 dpi (Table 2 and Fig. 6), with an average incubation period of 172.5 ± 5.2 days. Inoculation of all the animals with 139A-mouse PMCA-PrP^{res} induced the disease between 133 and 198 dpi (average, 169.8 ± 27.0 days). Surprisingly, all the mice infected with 263K-mouse PMCA-PrP^{res} showed typical symptoms between 131 and 180 dpi, with a comparable average incubation period (185.8 ± 19.2 days) similar to that in the above two groups. As expected, mice inoculated with 263K-hamster PMCA-PrP^{res} remained healthy until the end of the observation period (300 dpi). All the diseased animals showed similar clinical manifestations, with obvious weight loss and ataxia. Clinical course from the onset of symptoms to death was 5–13 days (8.0 ± 3.5) in animals infected with 139A-mouse PMCA-PrP^{res}, 3–11 days (7.5 ± 3.3) in animals infected with 263K-mouse PMCA-PrP^{res}, and 5–11 days (8.2 ± 2.6) in animals in the positive control group. As expected, all mice with clinical

Table 1 Clinical characteristics and brain PrP^{Sc} deposits of the hamsters inoculated with PMCA-PrP^{res}

Inoculums	No. of ill/ inoculated hamster	Incubation time (mean ± SD) (days)	Clinical course (mean ± SD) (days)	Major clinical manifestations				No. of PrP ^{Sc} positive/ inoculated hamster
				No. of positive/inoculated hamster				
				Weight loss	Ataxia	Sluggish	Tremble	
263K-hamster PMCA-PrP ^{res}	4/4	40 to 71 (56 ± 14.4)	3 to 11 (7.3 ± 3.5)	4/4	1/4	2/4	1/4	4/4
139A-hamster PMCA-PrP ^{res}	4/4	131 to 180 (157 ± 17.6)	6 to 13 (10.3 ± 3.1)	4/4	2/4	3/4	0/4	4/4
139A-mouse PMCA-PrP ^{res}	0/6	>300	>300	0/6	0/6	0/6	0/6	0/6
263K-infected hamster brain	4/4	65 to 77 (70.5 ± 4.93)	6 to 10 (7.0 ± 2.0)	6/6	2/6	4/6	2/6	6/6

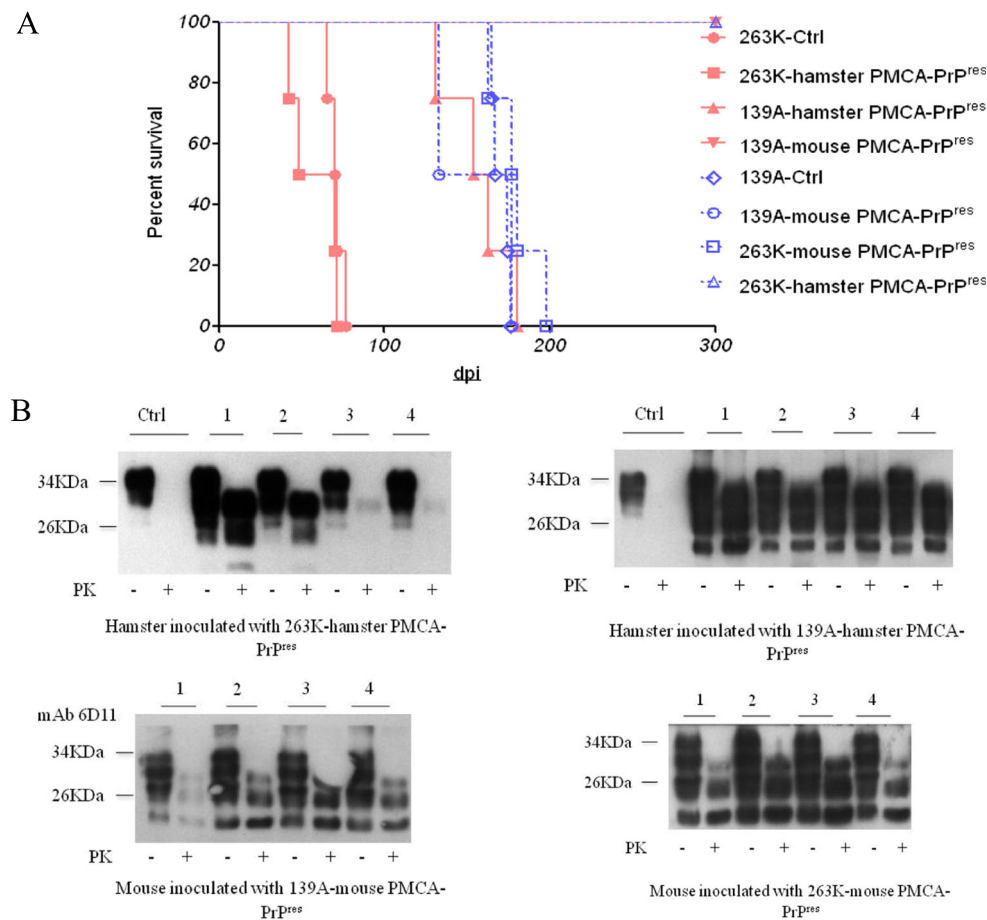


Fig. 6 Infectivities of newly generated hamster and mouse PMCA-PrP^{res} in experimental rodents. **a** Survival time. X-axis shows the survival time (days), and Y-axis shows the percentage of survival. Pink lines and dots represent hamsters inoculated with agent 263K seed (263K-Ctrl), PMCA product of 263K seed and hamster brain substrates (263K-hamster-PMCA-PrP^{res}), PMCA product of 139A seed and hamster brain substrates (139A-hamster-PMCA-PrP^{res}), or PMCA product of 139A seed and mouse brain substrates (139A-mouse-PMCA-PrP^{res}). Blue lines and dots represent mice inoculated with agent 139A seed (139A-

Ctrl), PMCA product of 139A seed and mouse brain substrates (139A-mouse-PMCA-PrP^{res}), PMCA product of 263K seed and mouse brain substrates (263K-mouse-PMCA-PrP^{res}), or PMCA product of 263K seed and hamster brain substrates (263K-hamster-PMCA-PrP^{res}). **b** Analyses of PK resistance of brain PrP^{Sc} in rodents inoculated with various PMCA products by performing western blotting. For this, 10 % of various brain homogenates were digested with 50 µg/ml PK and were blotted with mAb 6D11. Digital number represents different animals per group. Molecular weight standards are shown on the left

manifestations showed positivity for PrP^{res} in PK-treated western blots. PrP^{res} was detectable in the brains of mice inoculated with 263K-hamster PMCA-PrP^{res} (Table 2). Monoglycosylated PrP^{res} was significantly predominant in all the diseased mice brains. Quantitative assay of the ratios

of PrP^{res} signals showed that the percentages of di-, mono-, and un-glycosylated PrP^{res} were 37, 46, and 17 %, respectively, in animals infected with 263K-mouse PMCA-PrP^{res}; 33, 46, and 21 %, respectively, in animals infected with 139A-mouse PMCA-PrP^{res}; and 31, 41, and 28 %, respectively, in

Table 2 Clinical characteristics and brain PrP^{Sc} deposits of the mice inoculated with PMCA-PrP^{res}

Inoculums	No. of ill/ inoculated mice	Incubation time (mean ± SD) (days)	Clinical course (mean ± SD) (days)	Major clinical manifestations No. of positive/inoculated mice				No. of PrP ^{Sc} positive/ inoculated mice
				Weight loss	Ataxia	Sluggish	Tremble	
139A-mouse PMCA-PrP ^{res}	4/4	133 to 198 (169.8 ± 27.0)	5 to 13 (8.0 ± 3.5)	4/4	2/4	2/4	1/4	4/4
263K-mouse PMCA-PrP ^{res}	4/4	163 to 205 (185.8 ± 19.3)	3 to 11 (7.5 ± 3.3)	4/4	3/4	1/4	1/4	4/4
263K-hamster PMCA-PrP ^{res}	0/6	>300	>300	0/6	0/6	0/6	0/6	0/6
139A-infected mice brain	6/6	165 to 177 (172.5 ± 5.3)	5 to 11 (8.2 ± 2.6)	6/6	3/6	1/6	1/6	6/6

animals in the positive control group. These data suggested that the cross-species PrP^{res} generated using PMCA showed infectivity. These results also indicated that once the original scrapie agents changed their glycosylation patterns during cross-species propagation with PMCA, they maintained these features during subsequent infections in animals.

Discussion

Species barrier, a phenomenon through which an agent from one species can infect only a limited number of other species, is a typical feature of prion diseases [1]. As a zoonotic agent, the ability of various prion strains to be transmitted between different species has been used for evaluating their transmissibility among different species and for developing efficient experimental models [10, 11]. In addition to *in vivo* bioassays, barriers of interspecies prion transmission have been observed in many *in vitro* methodologies such as cell-free conversion and PMCA [12]. In the present study, we showed that PMCA helps hamster-adapted scrapie strain 263K and mouse-adapted scrapie strain 139A to convert normal PrP^C from opposite species to PrP^{res}. The newly generated cross-species PrP^{Sc} possesses the glycosylation characteristics of new hosts. Inoculation of these *in vitro* cross-species PMCA products successfully induces experimental TSEs in new hosts.

Our interspecies PMCA data are consistent with those of bioassays for prion infections, in which prions were more efficiently transmitted between homologous or heterologous animals with a close species relationship. When brain homogenates were used as the substrate, both the strains 263K and 139A induced an obvious conversion of homologous PrP^C during PMCA with fewer number of cycles (48 cycles) but showed a detectable conversion of heterologous PrP^C during PMCA with more number of cycles (144 cycles). The molecular basis of this process is not well understood. However, it is thought to be controlled by differences in amino acid sequences of PrP proteins [13, 14]. Notably, prion transmission barrier is not always symmetrical [15]. A good example of this is that inoculation of mouse-derived prion material in Syrian hamsters easily results in experimental scrapie [10] while transmission of infectious material from Syrian hamsters to mice is extremely difficult [16]. One of the explanations for this challenging transmission from hamsters to mice is the slow replication of prions in the mouse brain, which extends beyond the lifespan of the mouse and thus is a barrier in bioassays. However, this barrier can be successfully overcome by using PMCA, as observed in our experiments. Propagation of hamster prions with mouse-derived PrP^C can be stably achieved after long-term amplification. It seems that the significantly accelerated effect of PMCA has made it possible to replicate hamster prions within a short time in preparations containing heterologous mouse PrP^C, which could not be achieved with classical bioassays.

Similar to our previous study of cross-species infection with mouse-adapted agent 139A in hamsters [10], the glycosylation pattern of PMCA-generated 139A PrP^{Sc} in hamster tissue homogenates changed to a new host-associated pattern, with diglycosylated form being the most predominant. In addition, the glycosylation pattern of PMCA-generated 263K PrP^{Sc} in mouse tissue homogenates changed to a mouse-associated pattern, with monoglycosylated form being the most predominant. We also noted that these molecular traits were maintained stably in PrP^{Sc} identified in the brains of animals inoculated with interspecies PMCA-PrP^{res}, suggesting the formation of new prion strains during interspecies PMCA. The glycosylation pattern of PrP^{Sc} is one of the hallmarks for distinguishing between prion strains. In addition, studies have reported alterations in the biological and biochemical characteristics during passage in foreign species [3, 17], suggesting that interspecies transmission of prions, especially in wild-type animals containing endogenous PrP^C, acquires the features of new host.

The similarity in changes in the glycosylation profiles of PrP^{Sc} between cross-species PMCA and infectious bioassays strongly suggested that prion amplification during PMCA precisely copied the properties of prion replication in host tissues. In contrast, a BSE agent does not lose its original properties when causing an infection in humans (vCJD), cats (feline spongiform encephalopathy), and other ungulates (exotic ungulate encephalopathy) [18]. The exact mechanism underlying the diversity among prion strains during interspecies transmission is unclear. Interactions among exogenous prion seeds, host PrP^C, and unknown factors in host microenvironments may determine the features of new prions during a cross-species infection.

The major biochemical characteristics of interspecies PMCA-generated PrP^{Sc} (based on PrP^C of foreign species) seem to be fixed once new PrP^{Sc} is formed. These characteristics, including glycosylation profiles, PK resistance, and molecular weights that may represent protease-digesting sites in PrP^{Sc}, are stably passed during subsequent serial PMCA passages with same substrates. This observation is consistent with the interspecies transmission of mouse-adapted strains 139A and ME7 to hamsters in our previous study [10], with their serial passages in hamsters [19], and with other experimental and natural interspecies transmission of prions [17, 20]. These studies highlight the common and fundamental characteristics of prions. In addition, these studies suggest that the interface of seed PrP^{Sc} and substrate PrP^C in the early stage is one of the most essential processes for imposing the phenotype of a new prion strain. Interestingly, PrP^{Sc} molecules from earlier rounds of serial PMCA showed relatively lower PK resistance than those from later rounds, suggesting that the newly formed prions gradually stabilized their phenotypes during PMCA passages.

In line with the results of other studies [21], we confirmed that interspecies PMCA products (139A-hamster PMCA-

PrP^{res} and 263K-mouse PMCA-PrP^{res}) showed *in vivo* infectivity against new hosts. However, homologous PMCA products (139A-mouse PMCA-PrP^{res} and 263K-hamster PMCA-PrP^{res}) showed no infectivity against the opposite animals during the observation period. This indicates that the property of substrate PrP^C in tissue homogenates but not PMCA itself determines the interspecies infectivity of PMCA products. Incubation period for the infection of hamsters with 139A-hamster PMCA-PrP^{res} was longer than that for the infection of hamster with 263K-hamster brain strain and 263K-hamster PMCA-PrP^{res}, but was comparable to that for the infection of mice with 139A-mouse brain strain. In contrast, incubation period for the infection of mice with 263K-mouse PMCA-PrP^{res} was comparable to that for the infection of mice with 139A-mouse brain strain and 139A-mouse PMCA-PrP^{res} but was longer than that for the infection of mice with 263K-hamster brain strain. Unlike the interspecies transmission of scrapie-infected brains, with extremely long incubation period for primary infection but with short incubation period for successive passages [10], incubation period for PMCA-generated interspecies prions was stable during the second passage in the same hosts. This indicated that the infectious traits of the new interspecies prions were formed and stabilized during PMCA. However, it is still unclear why the incubation period for mouse-adapted agent 139A for infection in hamsters (inoculated with infected mouse brain homogenates or interspecies PMCA products) was similar to that for infection in mice while that of hamster-adapted agent 263K for infection in mice was longer than its original short incubation period.

In summary, in this study, we have in parallel evaluated the interspecies replication abilities of scrapie strains with PMCA *in vitro* and their interspecies infectivities *in vivo*. We confirmed that with the help of PMCA, both mouse- and hamster-adapted scrapie agents can sufficiently propagate with the opposite animal brains and muscles and cause experimental scrapie in opposite animals. Cross-species transmission of TSEs has been a great threat to public health and food safety since the outbreaks of BSE in cattle and vCJD in humans. Besides of prion strain, there are other factors that may determine or influence the breakthrough of the species barrier for the transmission of TSEs. Our data here may provide useful evidences that external environment factors can facilitate an exotic prion to infect a new specie host.

Materials and Methods

Preparation of Brain and Muscle Homogenates

Healthy 4-week-old male hamsters and mice were perfused with cold phosphate-buffered saline (PBS) plus 5 mM EDTA before harvesting the brain and muscle tissues. The brain and thigh muscles were removed surgically. Muscle tissues were

removed carefully to avoid contamination with peripheral nerves. Whole brain and thigh muscles were washed thoroughly with 10 volumes of cold 1× PBS containing 50 mM EDTA to remove blood before homogenization in PMCA conversion buffer (PBS containing 150 mM NaCl, 1.0 % Triton X-100, 5 mM EDTA, and protease inhibitor cocktail set III from Calbiochem). Crude homogenates were briefly centrifuged for 30 s at 3000×g by using an Eppendorf centrifuge. Aliquots of the supernatant were immediately frozen at 80 °C for subsequent experiments.

Brain tissues of Chinese hamsters inoculated intracerebrally with hamster-adapted scrapie agent 263K and those of C57BL/6 mice inoculated intracerebrally with mouse-adapted scrapie strain 139A were used. Incubation periods for 263K-infected hamsters and 139A-infected mice were 66.7±11 and 183.9±23.1 days, respectively [10]. Frozen scrapie agent 263K-infected hamster brains and 139A-infected mouse brains were homogenized in the PMCA conversion buffer, according to the procedure described above.

In Vitro Amplification by Performing PMCA

PMCA was performed in a water bath sonicator (Misonix sonicator 3000 and 4000; Misonix, Farmingdale, NY, USA) containing a microplate horn for PCR tubes. To avoid cross-contamination, PMCA, distribution of brain or muscle homogenates, addition of prion seeds, and western blotting were performed in separate rooms. Aliquots of scrapie strain 263K- or 139A-infected brain homogenates were diluted using 10 % brain or muscle homogenates of healthy mice or hamsters, respectively. Thin-walled 0.2-ml PCR tubes containing 100 µl of the mixed sample were placed in a floating position in the sonicator. Amplification cycles were programmed as described elsewhere [22]. One PMCA cycle included sonication at 90 % potency for 20 s, followed by incubation at 37 °C for 29 min and 40 s. In this study, one round of PMCA included 144 cycles. Direct PMCA for one round was conducted by mixing 10 µl of different concentrations of scrapie agents (as seed) with 90 µl of 10 % fresh normal homogenates (as substrate). For serial PMCA, 10 µl of amplified product after each round was added to 90 µl of fresh normal homogenates (10-fold dilution). The new mixture was then subjected to another round of PMCA. This procedure was repeated until the designated dilution was achieved.

Animal Bioassay

For this, 10 % infected rodent brain homogenates were mixed with 10-fold 10 % tissue homogenates of opposite normal animals and were subjected to 144 cycles of PMCA. The products were mixed with 10-fold individual normal tissue homogenates and were subjected to the next round of PMCA until the final dilution of the original scrapie agents

reached 10^{-17} . These PMCA products were used as inocula in the bioassay.

Twenty-eight-day-old golden hamsters or C57BL/6 mice were used for performing bioassays. The animals were grouped and were separately infected with four different inocula as follows. Hamsters were infected with inocula containing the PMCA product of agent 263K amplified with hamster brain homogenates, PMCA product of agent 139A amplified with hamster brain homogenates, PMCA product of agent 139A amplified with mouse brain homogenates, and brain homogenates of 263K-infected hamsters. Mice were infected with inocula containing the PMCA product of agent 139A amplified with mouse brain, PMCA product of agent 263K amplified with mouse brain, PMCA product of agent 263K amplified with hamster brain, and brain homogenates of 139A-infected mice. Each group included four to six hamsters or mice. The animals were intracerebrally injected with 10 μ l of the inocula (for hamsters) or 5 μ l inocula (for mice) under halothane anesthesia, according to protocols described elsewhere [19].

The animals were monitored twice a week before the appearance of clinical symptoms by experienced staff and once per day after the appearance of clinical symptoms until their death. Clinical symptoms of these animals were noted. Incubation period was calculated from inoculation to the onset of clinical manifestations, and clinical course was calculated from the onset of clinical manifestations to death at the terminal stage of the disease. At the end of the clinical phase, the animals were euthanized using ether and their brains were surgically removed for performing further analysis.

PK Degradation Assay

PK was purchased from Merck (Darmstadt, Germany). Lyophilized PK was dissolved in PK buffer containing 20 mM Tris (pH 7.5) and 1 mM CaCl_2 . Tissue homogenates or PMCA products were incubated with different concentrations of PK (0–1000 μ g/ml) at 37 °C for 60 min. Digestion was stopped by adding electrophoresis sample buffer and by boiling at 100 °C for 10 min. PK-resistant PrP was evaluated by performing western blotting.

Western Blotting

Scrapie strain 263K- or 139A-infected tissue homogenates and PMCA products were fractionated by performing 15 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were electrotransferred onto nitrocellulose membranes. After blocking with 5 % nonfat-dried milk in PBS, the membranes were incubated overnight at 4 °C with PrP-specific mAb (1:5000 dilution), including mAb 3F4 (Millipore, Billerica, MA, USA) and mAb 6D11, at room temperature for 2 h. After washing with PBS (containing 0.1 % Tween-

20; pH 7.6), the membranes were incubated with horseradish peroxidase-conjugated anti-mouse antibody (1:5000 dilution). Immunoreactive signals were visualized using an enhanced ECL kit (PE Applied Biosystems, Foster City, CA, USA).

PrP^{Sc} Quantification

Quantitative analysis of immunoblotting images was performed using ImageJ software. Values of each target blot were evaluated, and average values were calculated from three independent blots. All data are presented as mean \pm SD. Student's unpaired *t* test for the comparison of means was used for comparing the groups. Differences were considered significant at $P < 0.05$. All statistical analyses were performed using SPSS 17.0 statistical package.

Ethics Statement

Use of animal specimens in this study was approved by the Ethical Committee of the National Institute for Viral Disease Prevention and Control, Chinese Center for Disease Control, under protocol 2009ZX10004-101. All experimental rodents were maintained in a clean grade laboratory. Housing and experimental protocols were performed in accordance with the Chinese Regulations for the Administration of Affairs Concerning Experimental Animals.

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