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Species-Dependent Differences in Cofactor Utilization for Formation of the Protease-Resistant Prion Protein in Vitro†

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Abstract

The cofactor preferences for in vitro propagation of the protease-resistant isoforms of the prion protein (PrP^{Sc}) from various rodent species were investigated using the serial protein misfolding cyclic amplification (sPMCA) technique. Whereas RNA molecules facilitate hamster PrP^{Sc} propagation, RNA and several other polyanions do not promote the propagation of mouse and vole PrP^{Sc} molecules. Pretreatment of crude Prnp^{0/0} (PrP knockout) brain homogenate with RNase A or micrococcal nuclease inhibited hamster but not mouse PrP^{Sc} propagation in a reconstituted system. Mouse PrP^{Sc} propagation could be reconstituted by mixing PrP^C substrate with homogenates prepared from either brain or liver, but not from several other tissues that were tested. These results reveal species-specific differences in cofactor utilization for PrP^{Sc} propagation in vitro and also demonstrate the existence of an endogenous cofactor present in brain tissue not composed of nucleic acids.

Prions are the unconventional infectious agents of transmissible spongiform encephalopathies such as Creutzfeldt-Jakob disease (CJD),¹ scrapie, and chronic wasting disease (CWD) (1). During the course of such diseases, a membrane-bound glycoprotein expressed primarily in neurons termed PrP^C is converted by an unknown mechanism into an aggregated and frequently protease-resistant conformer, which has been designated PrP^{Sc} (1). The PrP^{Sc} conformer and prion infectivity have been amplified and propagated indefinitely in vitro using an intermittent sonication-based technique termed serial protein misfolding cyclic amplification (sPMCA), in which the products of one round of in vitro conversion are diluted and used as seeds to template successive conversion rounds (2–4). The chemical factors required for successful amplification and serial propagation of PrP^{Sc} molecules and prion infectivity in vitro have not yet been fully characterized. Studies

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SUPPORTING INFORMATION AVAILABLE

SDS-PAGE of the immunopurified PrP^C substrate, Western blot showing cofactor requirement for maintenance of PrP^{Sc} propagation, Western blot showing the effect of RNA on Hyper PrP^{Sc} propagation, Western blot showing the effect of GAGs on RML PrP^{Sc} propagation, agarose gel analysis of RNase activity, SDS-PAGE analysis of thermolysin activity, and Western blots showing the effect of proteases on PrP^{Sc} propagation. This material is available free of charge via the Internet at <http://pubs.acs.org>.

¹Abbreviations: PrP, prion protein; PrP^{Sc} or PrP-res, protease-resistant isoform; PrP^C, cellular isoform; sPMCA, serial protein misfolding cyclic amplification; CJD, Creutzfeldt-Jakob disease; CWD, chronic wasting disease; poly(A), polyadenylic acid; mAb, monoclonal antibody; CHO, Chinese hamster ovary; HaPrP, hamster prion protein; MoPrP, mouse prion protein; PVPrP, prairie vole PrP; IMAC, immobilized metal affinity chromatography; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; GAG, glycosaminoglycan; RNase, ribonuclease; PAGE, polyacrylamide gel electrophoresis; Tg, transgenic; Alb, albumin promoter.

utilizing the Sc237 strain of hamster scrapie showed that selective degradation of single-stranded RNA molecules inhibited PrP^{Sc} amplification in crude homogenates (5), which could subsequently be reconstituted by re-addition of RNA or other polyanions (5,6). The ability of RNA molecules to facilitate the propagation of hamster prions was confirmed when prions infectious for wild-type hamsters were generated de novo from a substrate preparation containing PrP^C and copurified lipid molecules supplemented with synthetic poly(A) RNA (3). Additional studies revealed that RNA molecules are selectively incorporated into nuclease-resistant complexes with hamster PrP molecules during prion formation in vitro and in situ (in large aggregates within the brains of scrapie-infected hamsters) (7), and treatment of brain homogenates with lithium aluminum hydride reduces hamster prion infectivity (8).

Prions can infect a wide variety of mammals, and interspecies transmission can produce infectious isolates with unique clinical and neuropathological features termed prion “strains” (9,10). Interestingly, there appear to be significant differences between various animal species in terms of their specific requirements for efficient PrP^{Sc} formation in vitro. For instance, whereas amplification of mouse PrP^{Sc} molecules in vitro requires the presence of an unglycosylated PrP^C substrate, amplification of hamster PrP^{Sc} molecules requires the presence of a glycosylated PrP^C substrate and is potently inhibited by unglycosylated PrP^C molecules in a dose-dependent manner (11). In this study, we have compared the cofactor preferences for efficient PrP^{Sc} propagation of several species and strains of rodent prions and discovered significant differences in the requirements for propagation of mouse and hamster prions in vitro.

MATERIALS AND METHODS

Reagents

Various prion strains used in this study were kindly provided by the following investigators: 22L and Hyper from S. Priola (National Institute of Allergy and Infectious Diseases, Bethesda, MD), Sc237 and RML from S. Prusiner (University of California, San Francisco, CA), and 301C from C. Soto (University of Texas, Houston, TX). Prnp^{0/0} mice were obtained from D. Harris (Boston University School of Medicine, Boston, MA) with the permission of C. Weissmann (Scripps Florida, Jupiter, FL). Prairie voles (*Microtus ochrogaster*) were obtained from C. Cramer (Dartmouth College, Hanover, NH). DNase-free RNase (11119915001) and micrococcal (S7) nuclease (107921) were both purchased from Roche (Indianapolis, IN). Thermolysin (88303), poly(A) RNA (P9403), trypsin (T1426), trypsin inhibitor (T9003), heparan sulfate (H9637), chondroitin sulfate (C4384), and dextran sulfate (D6924) were all purchased from Sigma (St. Louis, MO).

PrP^C Substrate Preparations

For RNA reconstitution experiments (Figure 1), the PrP^C substrate was immunopurified as previously described (3) with the following modifications. (1) Monoclonal antibody (mAb) 6D11 was substituted for mAb 3F4 as the capture antibody to permit binding to mouse and vole PrP^C. (2) The SP-Sepharose ion exchange chromatography step was omitted. (3) The neutralized 6D11 immunoaffinity column eluate was dialyzed against 20 mM MOPS (pH 7.5) and 0.15 M NaCl (molecular mass cutoff of 3.5 kDa) to yield the final product (Figure S1 of the Supporting Information). For experiments using Flp-In CHO cells (Invitrogen, Carlsbad, CA), HaPrP^C and MoPrP^C molecules were expressed and purified as previously described (7). For all other experiments, substrate preparations containing native PrP^C molecules were purified from frozen CD-1 mouse or Syrian hamster brains (Biochemed, Winchester, VA) using immobilized metal affinity and SP-Sepharose ion exchange

chromatography as previously described (11), substituting CoCl_2 for CuSO_4 to charge the IMAC column.

Tissue Homogenate Preparations

Homogenates of brain, liver, and lung were prepared from $\text{Prnp}^{0/0}$ mice perfused with phosphate-buffered saline without calcium or magnesium (PBS) with 5 mM EDTA. Tissues were Potter-homogenized in 9 volumes of PBS, and particulate debris was removed by centrifugation for 30 s at 200g. Residual blood was removed by further centrifugation at 10000g for 20 min at 4 °C. Pellets were then brought back to the original volume using PBS and rehomogenized with a Potter homogenizer.

Enzyme Treatments

Where indicated, $\text{Prnp}^{0/0}$ mouse brain homogenates were pretreated using the following protocols. In sPMCA experiments, all $\text{Prnp}^{0/0}$ brain homogenates used for positive control reactions were mock-incubated under identical conditions in the absence of enzyme. Digestion with DNase-free RNase was performed by incubation of 1.0 mL of brain homogenate with 1.5 units/mL enzyme for 1.0 h at 37 °C. Digestion with micrococcal nuclease was performed by incubation of 1.0 mL of brain homogenate with 15000 units/mL enzyme and 2.5 mM CaCl_2 for 1.0 h at 37 °C. Digestion with thermolysin was performed by incubation of 1.0 mL of brain homogenate with 1.0 unit/mL enzyme and 2 mM CaCl_2 for 1.0 h at 70 °C. Digestion with trypsin was performed by mixing 225 μL of brain homogenate with either 25 μL of 1mM HCl (control) or 25 μL of trypsin in 1mM HCl to a final trypsin concentration of 25 $\mu\text{g}/\text{mL}$. Samples were incubated at 25 °C on an end-over-end rotator for 1 h, and digestion was terminated by addition of 50 $\mu\text{g}/\text{mL}$ trypsin inhibitor.

Nucleic Acid Preparation

Total RNA was prepared from mouse brain using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. RNA quality was confirmed by monitoring OD_{260} and OD_{280} and by agarose gel electrophoresis. Genomic DNA was prepared by incubation of brain homogenate overnight at 50 °C in the presence of 5 mM EDTA, 200 mM NaCl, 0.2% SDS, and 0.2 mg/mL proteinase K (Roche). Undigested material was removed by centrifugation at 10000g for 5 min. The supernatant was transferred to a separate tube containing an equal volume of a phenol/chloroform/isoamyl alcohol mixture (25:24:1) (Amresco, Solon, OH). The sample was vortexed briefly and centrifuged at 10000g for 15 min. The resulting supernatant was transferred to a different tube containing roughly 2 volumes of 100% ethanol and 1/10 volume of sodium acetate (pH 5.5). Tubes were then inverted five or six times until DNA appeared and were subsequently centrifuged at 10000g for 10 min. Pellets were washed with 70% ethanol, centrifuged for an additional 5 min at 10000g, air-dried, and resuspended in nuclease-free TE buffer (pH 8.0) (Ambion, Austin, TX).

Serial Protein Misfolding Cyclic Amplification

Reconstituted sPMCA reactions, adapted from the method of Castilla et al. (2), were performed as previously described (12), using 24 h rounds, 30 min cycles, 30 s pulses, and power settings between 70 and 85%. Day 1 reaction mixtures were seeded with 10 μL of 1% (w/v) scrapie brain homogenate in PBS with 1% Triton X-100. It should be noted that the percent conversion varies between individual experiments and is notably higher for hamster PrP than mouse PrP substrate. The readout for sPMCA reactions is maintenance rather than amplification of PrP^{Sc} levels over multiple rounds. PrP^{Sc} propagation in reconstituted reaction mixtures is dependent upon the presence of cofactors such as $\text{Prnp}^{0/0}$ brain

homogenate and stops when such cofactors are removed (Figure S2 of the Supporting Information).

PrP^{Sc} Detection

To detect PrP^{Sc} molecules, hamster samples were digested with 50 $\mu\text{g}/\text{mL}$ proteinase K for 1 h. at 37 °C, and mouse and vole samples were digested with 25 $\mu\text{g}/\text{mL}$ proteinase K for 30 min at 37 °C. All samples were processed for SDS–PAGE and Western blotting as previously described (12), using mAb 6D11 (whose epitope is QWNK, corresponding to residues 97–100 of MoPrP) as the primary antibody. In each blot, the positions of three prestained molecular mass markers (37, 24, and 17 kDa unless otherwise stated) are indicated by horizontal lines.

RESULTS

RNA Does Not Facilitate Propagation of Mouse and Vole PrP^{Sc} Molecules

We previously demonstrated that protease-resistant PrP^{Sc} molecules and infectious prions could be propagated by sPMCA in vitro using a substrate mixture containing Syrian hamster (Ha) PrP^C and poly(A) RNA molecules (3). PrP^{Sc} molecules can also be propagated in sPMCA reaction mixtures seeded with the Sc237 strain of hamster scrapie by using a substrate mixture of HaPrP^C molecules supplemented with a preparation of total mouse brain RNA (Figure 1A). Similar results were obtained using the Hyper strain of hamster scrapie (Figure S3 of the Supporting Information).

To test whether in vitro propagation of mouse PrP^{Sc} molecules can also be facilitated by RNA, we performed sPMCA reaction with mixtures seeded with the RML strain of murine scrapie using a substrate mixture of mouse PrP^C (MoPrP^C) molecules and a preparation of total mouse brain RNA. The results indicate that total brain RNA did not facilitate the propagation of mouse RML PrP^{Sc} molecules in vitro (Figure 1B, bottom blot). In contrast, MoPrP^C reconstituted with Prnp^{0/0} (PrP knockout) brain homogenate successfully propagates mouse RML PrP^{Sc} molecules (Figure 1B, top blot), confirming that our preparation of MoPrP^C substrate is competent for conversion into the PrP^{Sc} conformation. Total mouse brain RNA failed to facilitate the propagation of RML PrP^{Sc} molecules when tested at varying concentrations between 1 and 100 $\mu\text{g}/\text{mL}$, as did synthetic poly(A) RNA and genomic DNA (data not shown). The glycosaminoglycan (GAG) molecules heparan sulfate, chondroitin sulfate, and dextran sulfate also failed to support the propagation of RML PrP^{Sc} molecules (Figure S4 of the Supporting Information).

To distinguish whether the inability of RNA and the other polyanions tested to facilitate mouse RML PrP^{Sc} propagation was attributable to the change in PrP^C species or to the prion strain, we investigated whether total mouse brain RNA could facilitate the propagation of Sc237 PrP^{Sc} molecules using prairie vole PrP^C (PVPrP^C) as a substrate. Whereas MoPrP^C is not an efficient substrate for hamster prions in sPMCA reactions (13), PVPrP^C successfully propagates Sc237 PrP^{Sc} molecules in the presence of Prnp^{0/0} brain homogenate (Figure 1C, top blot). However, Sc237 PrP^{Sc} molecules could not be propagated when the PVPrP^C substrate was combined with total mouse brain RNA (Figure 1C, bottom blot). This result indicates that the differences in cofactor usage observed in these experiments can be attributed to amino acid sequence differences between the PrP^C molecules of the three rodent species, rather than differences in prion seeds.

RNA Molecules Are Not Required for Propagation of Mouse PrP^{Sc} Molecules

We next investigated whether species-specific differences in cofactor usage might exist when PrP^{Sc} molecules are propagated in the presence of crude brain homogenate. In

previous work, we observed that treatment of crude brain homogenates with nucleases that degrade single-stranded RNA molecules inhibited the amplification of Sc237 HaPrP^{Sc} molecules (5). Similarly, pretreatment of a Prnp^{0/0} brain homogenate with ribonuclease (RNase) inhibits reconstituted Sc237 HaPrP^{Sc} sPMCA propagation, confirming that RNA is the predominant cofactor within crude brain homogenate utilized for hamster PrP^{Sc} formation *in vitro* (Figure 2A). In contrast, RNase pretreatment did not affect the reconstituted propagation of MoPrP^{Sc} molecules in sPMCA reaction mixtures seeded with RML (Figure 2B), 301C (Figure 2C), or 22L (Figure 2D) prions. It should be emphasized that the same preparation of RNase-pretreated Prnp^{0/0} brain homogenate was used for both hamster and mouse reconstitution experiments, and thus, the inhibition of hamster PrP^{Sc} amplification serves as a functional internal control for effective RNase digestion in these assays. In addition, analysis of Trizol-extracted material by agarose gel electrophoresis confirmed the lack of detectable RNA molecules in RNase-treated brain homogenate (Figure S5 of the Supporting Information). Taken together, these results suggest that RNA is neither a cofactor nor an inhibitor of MoPrP^{Sc} propagation in sPMCA reactions.

Pretreatment of Prnp^{0/0} brain homogenate with micrococcal (S7) nuclease, which digests DNA as well as RNA molecules, also did not inhibit the reconstituted propagation of mouse PrP^{Sc} molecules (Figure 3, bottom blot), indicating that neither ribonucleic acids are required for sPMCA propagation of mouse PrP^{Sc} molecules (Figure 3, bottom blot). As an internal control, we tested the ability of the same preparation of micrococcal nuclease-treated Prnp^{0/0} brain homogenate to reconstitute HaPrP^{Sc} propagation; the results of this experiment confirmed that propagation of HaPrP^{Sc} was unsuccessful in the absence of nucleic acids, as expected (Figure 3, top blot).

PrP Sequence Determines Cofactor Preference

To rule out the possibility that the observed differences in cofactor preference might be attributable to the presence of species-specific molecules that may have hypothetically copurified with PrP^C during substrate preparation, we expressed the genes encoding HaPrP^C and MoPrP^C in Flp-In Chinese hamster ovary (CHO) cells. As previously described, site-directed integration into Flp-In cells ensures that the two species of PrP molecules are produced on an identical genetic background, and CHO-expressed, recombinant PrP^C substrate molecules can propagate PrP^{Sc} molecules and infectious prions *in vitro* (14). Using this system, we tested the ability of poly(A) RNA to facilitate the conversion of CHO-expressed HaPrP^C and MoPrP^C substrates in sPMCA reaction mixtures seeded with Sc237 hamster and RML mouse prions, respectively. The results indicate that RNA successfully facilitates formation of PrP^{Sc} molecules from CHO-expressed HaPrP^C but not CHO-expressed MoPrP^C substrate, whereas Prnp^{0/0} brain homogenate facilitates the formation of PrP^{Sc} from both substrates (Figure 4). Note that the relatively slow electrophoretic mobility of CHO-expressed PrP molecules can be attributed to hyperglycosylation (14). These results show that the differential ability to utilize RNA as a propagation cofactor can be directly attributed to differences in the amino acid sequence between mouse and hamster PrP molecules.

Conversion Cofactor Is Protease-Resistant and Heat-Stable

It has been previously proposed that the formation of mouse prions *in vivo* requires an accessory “protein X”, which was also hypothesized to mediate dominant negative inhibition by various polymorphic PrP molecules (15–18). Furthermore, some specific protein chaperones and cellular receptors have been shown to promote PrP^{Sc} formation *in vitro* (19,20). To investigate the possibility that the non-nucleic acid activity that facilitates mouse PrP^{Sc} propagation might be proteinaceous, we tested the ability of Prnp^{0/0} brain homogenate pretreated with thermolysin protease to reconstitute PrP^{Sc} propagation. The

results of this experiment show that protease digestion did not inhibit the propagation of either hamster or mouse PrP^{Sc} molecules (Figure 5). SDS-PAGE analysis confirmed that thermolysin treatment degraded the large majority of brain proteins originally present in the crude homogenate, but it should be noted that some proteins apparently resisted protease digestion (Figure S6 of the Supporting Information). It should also be noted that digestion with thermolysin was performed for 1 h at 70 °C. Therefore, these experiments also demonstrate that the cofactor responsible for facilitating MoPrP^{Sc} propagation is heat-stable. Similar results were also obtained using trypsin (Figure S7 of the Supporting Information) or a combination of thermolysin and trypsin (Figure S8 of the Supporting Information).

Tissue Distribution of Cofactor Activity

Finally, we investigated the distribution of the activity responsible for facilitating MoPrP^{Sc} propagation. We dissected the brain, kidney, liver, lung, and spleen from a saline-perfused Prnp^{0/0} mouse and prepared homogenates from all three organs. These homogenates were then used to reconstitute MoPrP^C substrate in sPMCA reaction mixtures seeded with RML mouse prions. The results of these reconstitution experiments indicate that both brain and liver homogenate facilitate the propagation of RML-seeded mouse PrP^{Sc} molecules, but the other tissues do not (Figure 6).

DISCUSSION

The major finding reported here is that cofactor preferences for in vitro PrP^{Sc} propagation differ for PrP^C substrate molecules derived from different animal species. Specifically, our results indicated that, whereas hamster PrP^{Sc} propagation preferentially utilizes RNA as a cofactor in both purified reactions and crude homogenate mixtures, RNA fails to facilitate either mouse or vole PrP^{Sc} propagation. Experiments using hamster Sc237 prions to seed both hamster and vole PrP^C substrates in sPMCA reaction mixtures, as well as a screen of three different mouse prion strains in RNase sensitivity experiments, confirmed that the observed cofactor preference is dependent upon PrP^C species rather than prion strain.

By expressing both hamster and mouse PrP^C molecules in Flp-In CHO cells, we were able to generate substrate preparations from an identical cellular source and thereby confirm that differential cofactor preference must be caused by the differences in amino acid sequence between hamster and mouse PrP. There are 11 amino acid differences between the sequences of the mature, post-translationally processed Syrian hamster and mouse PrP^C molecules, which are distributed throughout the protein. Additional work with recombinant, chimeric molecules will be required to identify specifically the subset of these variant residues that enable hamster PrP^C to use RNA as a propagation cofactor.

Because sPMCA is an in vitro technique, it is certainly not possible to conclude from our results that different animal species utilize different cofactors when propagating prions in vivo. However, if such a situation did exist, it could potentially provide a simple explanation for the observation that Syrian hamsters appear to develop scrapie more quickly than mice following infection. Alternatively, it is possible that PrP^C molecules from all animal species utilize the same universal cofactor during prion propagation, but that some PrP^C species (such as hamster PrP^C) may be inherently more promiscuous and therefore able to utilize a variety of different cofactors to facilitate PrP^{Sc} propagation in vitro. Within this scenario, promiscuous PrP^C species could potentially recruit additional cofactors as the disease progresses, thereby accelerating PrP^{Sc} accumulation. The recruitment of potent intracellular cofactors such as RNA might be made possible by prion-induced cellular damage compromising the integrity of membrane compartmentalization. Ultimately, transmission studies in genetically modified hosts will be required to confirm the role of putative cofactor molecules in prion infection in vivo.

On the basis of the results of our studies, the cofactor activity that facilitates propagation of mouse PrP^{Sc} molecules in vitro does not appear to be a nucleic acid. The activity is also unlikely to be mediated by proteins, due to the lack of an effect on reconstituted MoPrP^{Sc} propagation of protease digestion and heat treatment, which hydrolyze and denature most proteins, respectively. Among the known remaining classes of cellular macromolecules, the greatest amount of existing evidence supports potential roles for GAG and lipid molecules as potential prion cofactors. GAGs stimulate HaPrP-res formation in vitro (21) and assist in the recovery of infectivity during refolding of denatured hamster prions (22). Inhibitors of GAG synthesis as well as GAG-degrading enzymes reduce the level of formation of MoPrP^{Sc} in cultured ScN2a cells (23). However, GAGs are linear polyanions that resemble single-stranded nucleic acids structurally, and like RNA, heparan sulfate failed to facilitate MoPrP^{Sc} propagation in our assays. Noncovalently attached lipid molecules can be detected in detergent-extracted, purified preparations of both PrP^C and PrP^{Sc} molecules (24). Dispersal of hamster prion rods into phospholipid liposomes increases infectivity between 10- and 100-fold (25). Disruption of lipid metabolism influences MoPrP^{Sc} levels in ScN2a cells (26,27), and isolated lipid rafts support in vitro PrP^{Sc} formation (28). Phospholipids promote the conversion of recombinant PrP molecules into a PrP^{Sc}-like conformation that is enriched in β -sheet structure and partially protease-resistant (29,30). The activity responsible for facilitating MoPrP^{Sc} propagation in vitro appears to be present in both brain and liver. It has been previously reported that ectopic expression of MoPrP^C in hepatocytes did not facilitate prion replication in the livers of healthy Tg(Alb-PrP) transgenic mice (31). However, the PrP^C expression levels in the livers of Tg(Alb-PrP) mice were significantly lower than the PrP^C levels in the brains of control mice (31), and Tg(Alb-PrP) mice with chronic hepatic inflammation did replicate infectious prions in their livers (32), suggesting that hepatocytes contain all the cellular factors required to replicate prions. More work is required to characterize, purify, and identify the molecules in brain and liver homogenates responsible for facilitating MoPrP^{Sc} propagation in vitro.

Recently, in a major advance, Wang et al. demonstrated that infectious recombinant mouse prions could be formed de novo from a mixture of recombinant MoPrP, 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG), and total liver RNA molecules (33). One possible explanation for these apparently conflicting results is that either POPG alone or the combination of POPG and RNA can mimic the endogenous cofactor in brain homogenate. It is also possible that native and recombinant PrP substrates may differ in their requirements for prion formation.

One intriguing question raised by the discovery of multiple in vitro prion conversion cofactors is whether such compounds might be necessary for the maintenance of PrP^{Sc} structure. We previously found that single-stranded nucleic acids are selectively incorporated into nuclease-resistant complexes with PrP during the process of prion formation in vitro (7). Those results raise the possibility that various nonproteinaceous cofactors may help maintain PrP^{Sc} architecture, which in turn may encode infectivity and/or strain properties. It will be interesting to determine experimentally whether prions with high specific infectivity can be formed using only PrP, as predicted by the “protein only” hypothesis, or whether structural components other than PrP are required to generate an infectious complex.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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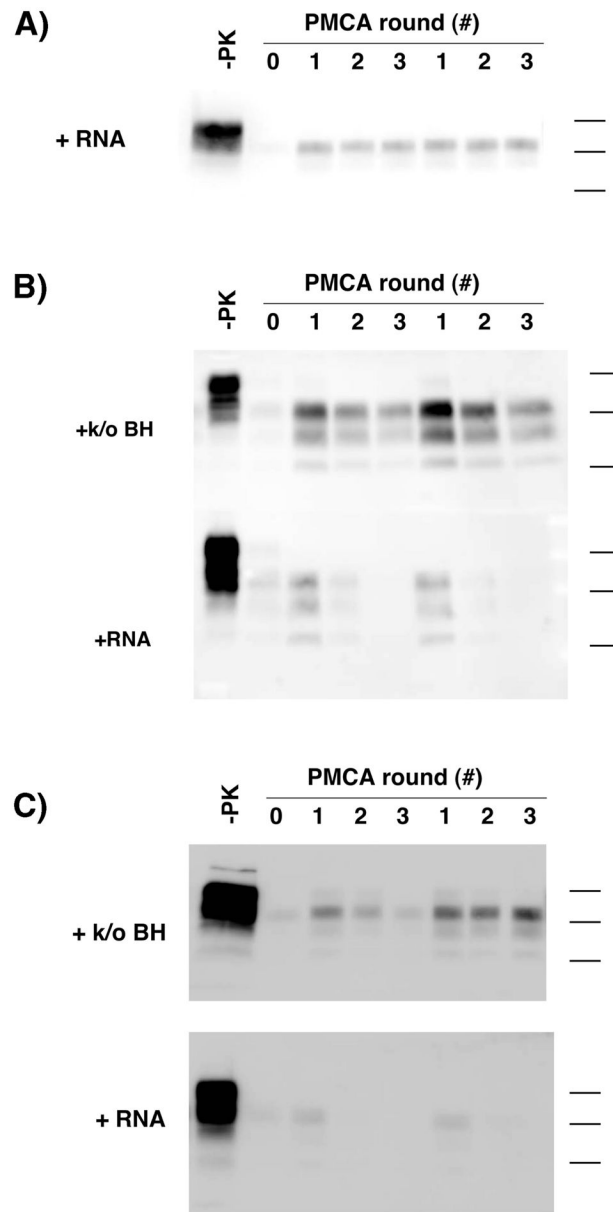


Figure 1. Effect of RNA on PrP^{Sc} propagation in vitro. Western blots showing sPMCA reactions using PrP^C substrates prepared from various rodent species: (A) hamster PrP^C seeded with Sc237 prions, (B) mouse PrP^C seeded with RML prions, and (C) prairie vole PrP^C seeded with Sc237 prions. In each blot, the first lane contained a sample not subjected to protease digestion to show the electrophoretic mobility of the input PrP^C substrate (-PK). All other samples were subjected to limited proteolysis with proteinase K. All blots show the results of two independent three-round sPMCA experiments. In each experiment, the round 0 sample is identical in composition to the corresponding round 1 samples but was not subjected to sPMCA. The PrP^C substrate molecules in each experiment were supplemented either with Prnp^{0/0} brain homogenate (+k/o BH) or with 10 μ g/mL total mouse brain RNA (+RNA), as indicated.

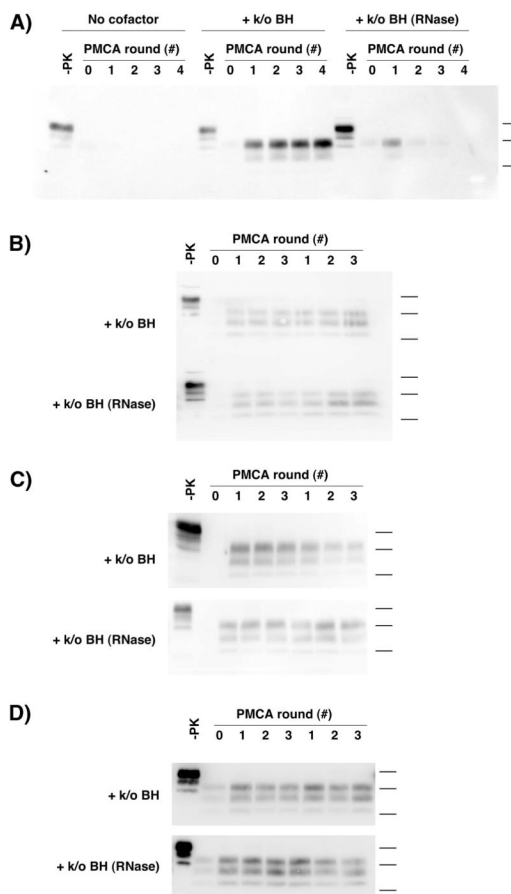


Figure 2. Effect of RNase on PrP^{Sc} propagation in vitro. Western blots showing reconstituted sPMCA reactions using either hamster or mouse PrP^C substrate, seeded with various prion strains: (A) HaPrP^C seeded with Sc237 prions, (B) MoPrP^C seeded with RML prions, (C) MoPrP^C seeded with 301C prions, and (D) MoPrP^C seeded with 22L prions. Where indicated, the PrP^C substrate molecules were supplemented with buffer (No cofactor), Prnp^{0/0} brain homogenate (+k/o BH), or RNase-pretreated Prnp^{0/0} brain homogenate (+k/o BH). The same preparation of RNase-treated Prnp^{0/0} brain homogenate was used for all of the experiments shown.



Figure 3.

Effect of micrococcal nuclease on PrP^{Sc} propagation in vitro. Western blots showing sPMCA reactions using either HaPrP^C substrate seeded with Sc237 prions (top) or MoPrP^C substrate seeded with RML prions (bottom), supplemented with Prnp^{0/0} brain homogenate. Where indicated, the Prnp^{0/0} brain homogenate was pretreated by digestion with micrococcal nuclease prior to sPMCA (+S7 Nuclease). The same preparation of micrococcal nuclease-treated Prnp^{0/0} brain homogenate was used for all of the experiments shown.

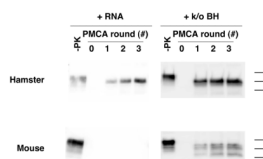


Figure 4.

Effect of RNA on CHO-expressed PrP^{Sc} propagation *in vitro*. Western blots showing sPMCA reactions using either HaPrP^C (top) or MoPrP^C (bottom) substrates expressed in Flp-In CHO cells, seeded with Sc237 or RML prions, respectively. The PrP^C substrate molecules in each experiment were supplemented either with Prnp^{0/0} brain homogenate (+k/o BH) or 10 μ g/mL poly(A) RNA (+RNA), as indicated. For each PrP^C species, results of two independent three-round sPMCA experiments supplemented with RNA are shown. Note that sPMCA mixtures supplemented with Prnp^{0/0} brain homogenate were analyzed on physically separate Western blots. Horizontal lines representing 43, 34, and 26 kDa are used to indicate molecular masses.

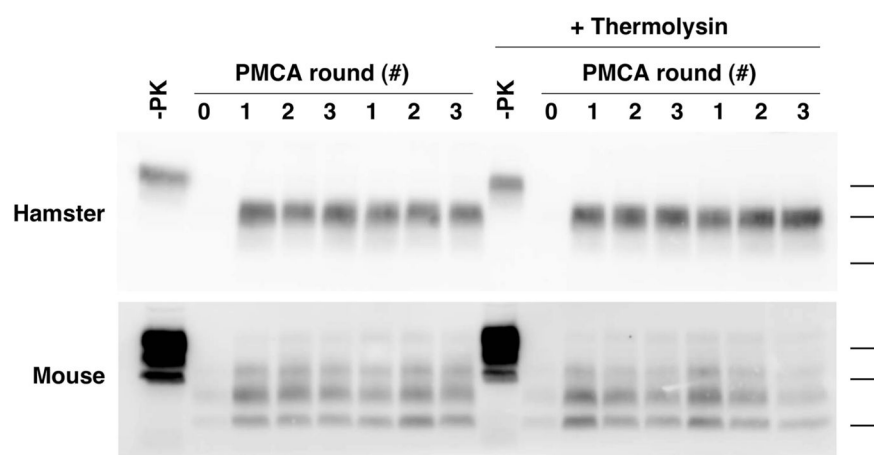


Figure 5. Effect of thermolysin on PrP^{Sc} propagation in vitro. Western blots showing sPMCA reactions using either HaPrP^C substrate seeded with Sc237 prions (top) or MoPrP^C substrate seeded with RML prions (bottom), supplemented with Prnp^{0/0} brain homogenate. Where indicated, the Prnp^{0/0} brain homogenate was pretreated by digestion with thermolysin prior to sPMCA (+Thermolysin).

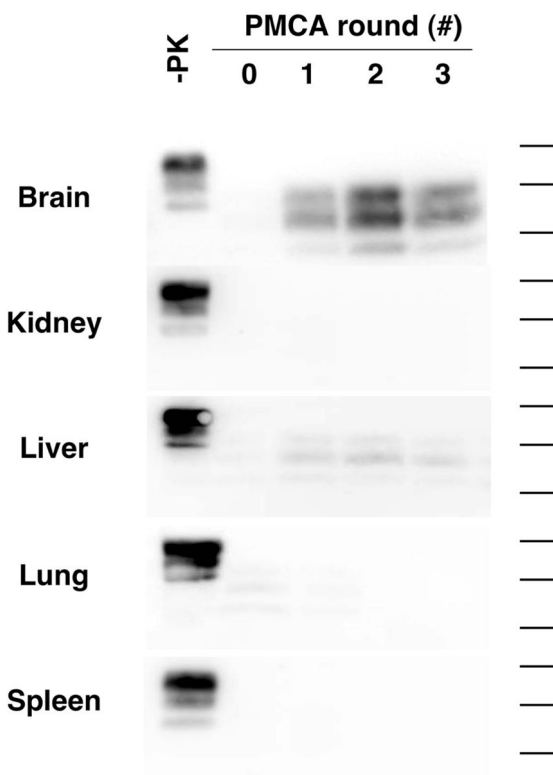


Figure 6. Ability of various tissue homogenates to reconstitute MoPrP^{Sc} propagation. Western blots showing sPMCA reactions using MoPrP^C substrate seeded with RML prions, reconstituted with tissue homogenates prepared from various tissues of a Prnp^{0/0} mouse, as indicated.