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# Isolation of phosphatidylethanolamine as a solitary cofactor for prion formation in the absence of nucleic acids

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**Infectious prions containing the pathogenic conformer of the mammalian prion protein ($PrP^{Sc}$) can be produced de novo from a mixture of the normal conformer ($PrP^{C}$) with RNA and lipid molecules. Recent reconstitution studies indicate that nucleic acids are not required for the propagation of mouse prions in vitro, suggesting the existence of an alternative prion propagation cofactor in brain tissue. However, the identity and functional properties of this unique cofactor are unknown. Here, we show by purification and reconstitution that the molecule responsible for the nuclease-resistant cofactor activity in brain is endogenous phosphatidylethanolamine (PE). Synthetic PE alone facilitates conversion of purified recombinant (rec)PrP substrate into infectious $recPrP^{Sc}$ molecules. Other phospholipids, including phosphatidylcholine, phosphatidylserine, phosphatidylinositol, and phosphatidylglycerol, were unable to facilitate $recPrP^{Sc}$ formation in the absence of RNA. PE facilitated the propagation of $PrP^{Sc}$ molecules derived from all four different animal species tested including mouse, suggesting that unlike RNA, PE is a promiscuous cofactor for $PrP^{Sc}$ formation in vitro. Phospholipase treatment abolished the ability of brain homogenate to reconstitute the propagation of both mouse and hamster $PrP^{Sc}$ molecules. Our results identify a single endogenous cofactor able to facilitate the formation of prions from multiple species in the absence of nucleic acids or other polyanions.**

PrP | scrapie

Prions are mechanistically unique infectious agents that contain a misfolded, membrane-bound, glycoprotein ($PrP^{Sc}$) formed by the conformational change of a host-encoded conformer ($PrP^{C}$) (1). Conversion of $PrP^{C}$ into $PrP^{Sc}$ is the central event in the formation of infectious prions, but the molecular mechanism underlying conformational change remains poorly understood. In particular, the number and identity of endogenous factors other than PrP required for prion formation has not been determined (2).

Cell culture and biochemical studies have implicated several classes of macromolecules such as GAGs, nucleic acids, proteins, and lipids as potential cofactors for prion formation (3). Reconstitution experiments with defined substrates (in which purified PrP molecules are mixed with $Prnp^{0/0}$ brain homogenate or purified cofactors that facilitate its conversion to $PrP^{Sc}$) have suggested that the conversion mechanism may be relatively simple, requiring only a few components (4, 5). Wild-type hamster prions possessing specific infectivity levels similar to those associated with natural scrapie have been formed de novo by using a defined mixture of purified native $PrP^{C}$, copurified lipid, and RNA molecules (4), and infectious prions have also been formed de novo from bacterially expressed, recombinant PrP substrate in a reaction facilitated by synthetic 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) and RNA molecules (5, 6). In summary, infectious prions have not yet been produced either with a single cofactor or in the absence of nucleic acids.

Recent studies using reconstitution experiments with purified $PrP^{C}$ molecules have shown that polyanions such as RNA molecules facilitate the propagation of hamster but not mouse prions (7). Furthermore, these experiments showed that $Prnp^{0/0}$ (PrP knockout) mouse brain homogenate contains a heat-stable activity capable of stimulating mouse prion propagation in vitro. Therefore, we sought to identify the novel molecule(s) responsible for this cofactor activity by biochemical purification using the reconstitution of mouse prion propagation as an assay.

## Results

Preliminary studies indicated that the cofactor activity responsible for facilitating mouse $PrP^{Sc}$ propagation in $Prnp^{0/0}$ brain homogenates was membrane bound and insoluble in the nonionic detergent Triton X-100 (Fig. S1, *Upper*). To solubilize the cofactor, we conducted a detergent screen, and found that the cofactor activity could be completely solubilized with n-octyl glucoside (NOG) (Fig. S1, *Lower*). We then exploited the heat , protease, and nuclease sensitivity of the cofactor activity (7) to develop a purification protocol from NOG-solubilized $Prnp^{0/0}$ mouse brain membranes as described in *Materials and Methods*.

To determine the substrate specificity of the endogenous cofactor, we tested the ability of the purified preparation to facilitate propagation of both mouse and hamster prions in reconstituted serial protein misfolding cyclic amplification (sPMCA) reactions by using immunopurified $PrP^{C}$ substrates. Unlike RNA, which facilitates in vitro propagation of hamster Sc237 but not mouse RML prions (7), our preparation of purified cofactor activity facilitates the propagation of both Sc237 and RML prions (Fig. 1*A*), suggesting that the endogenous cofactor may interact with a wider range of prions than RNA. In addition, the cofactor preparation also facilitates the propagation of deer and vole $PrP^{Sc}$ in the reconstituted sPMCA reactions (Fig. S2).

Wang et al. showed that mouse PrP recombinantly produced in *Escherichia coli* (recPrP) is a suitable substrate for sPMCA reactions facilitated by synthetic phosphatidylglycerol (PG) and total liver RNA cofactors (5). We tested the ability of our cofactor preparation to enable the conversion recPrP in sPMCA reactions without RNA. The results show that the cofactor preparation facilitates conversion of recPrP into an autocatalytic,

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Conflict of interest statement: S.S. and N.D. are inventors of a patent application that covers the use of phosphatidylethanolamine as a prion cofactor.

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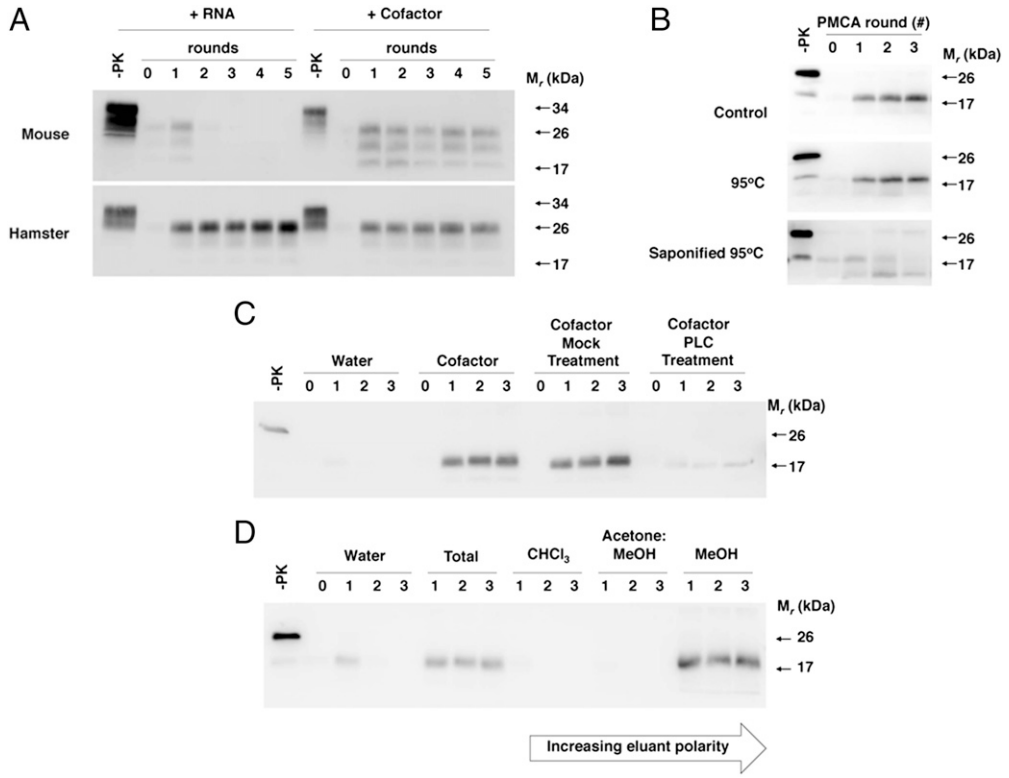


**Fig. 1.** Characterization of cofactor preparation. Western blots of reconstituted sPMCA reactions probed with 6D11 anti-PrP mAb. (*A*) Species specificity. Immunopurified native mouse $PrP^C$ substrate initially seeded with Me7 prions (*Upper*) or hamster $PrP^C$ substrate seeded Sc237 prions (*Lower*) were supplemented with either 50 μg/mL total rat brain RNA (*Left*) or a purified cofactor (*Right*). (*B* and *C*) Saponification and PLC treatment. Saponified and phospholipase-treated cofactor samples were used to reconstitute sPMCA reactions with recPrP substrate seeded with $recPrP^{Sc}$ template, as indicated. (*D*) Silica chromatography fractionation. recPrP substrate was supplemented with normal-phase chromatographic fractions and subjected to sPMCA reactions seeded with $recPrP^{Sc}$ template, as indicated. -PK, samples not subjected to proteinase K digestion; all other samples were proteolyzed.

protease-resistant conformation with an ~18-kDa core (Fig. 1*B, Top*).

The membrane association and detergent solubility of the cofactor activity, and the physical appearance of the purified preparation suggested that the endogenous cofactor might be a lipid molecule. To test this hypothesis, we examined the effect of saponification on cofactor activity. Our experiments showed that saponification by treatment with alkali at 95 °C destroyed the ability of the cofactor preparation to facilitate formation and propagation of $recPrP^{Sc}$ (Fig. 1*B, Lower*). Interestingly, in these sPMCA reactions, a different autocatalytic conformer with a ~14-kDa protease-resistant core emerged after two rounds (Fig. 1*B, Lower*). We used recPrP as substrate for all subsequent experiments because it could be produced more easily than purified native $PrP^C$ and also because purified $PrP^C$ contains stoichiometric amounts of copurified lipids that could confound our reconstitution experiments (4).

Within brain membranes, phospholipids are the major class of saponafiable compounds. To test whether one or more phospholipids might be responsible for the cofactor activity in our purified preparation, we treated the preparation with phospholipase C (PLC), an enzyme that specifically cleaves phospholipids at the bond between the glycerol backbone and phosphate. Our results indicated that PLC treatment inhibited the propagation of $recPrP^{Sc}$, indicating that the cofactor preparation contains an essential phospholipid (Fig. 1*C*).

Next, we used normal phase chromatography to separate our cofactor preparation into three fractions of increasing polarity, enriched in neutral lipids, sphingolipids, and phospholipids, respectively. This analysis revealed that the cofactor activity was only present in the most polar MeOH fraction, into which phospholipids partition (Fig. 1*D*).

We performed $^{31}$P-NMR to analyze the phospholipid composition of our purified preparation quantitatively. The results showed that our preparation contains phosphatidylethanolamine (PE), lysoPE, phosphatidylcholine (PC), lyso PC, and phosphatidylinositol (PI) (Table 1). PE is the most abundant phospholipid in our preparation, accounting for ~50% of the phospholipid in the sample, whereas phosphatidylserine (PS), phosphatidylglycerol (PG), and sphingolipids were not detected. Flow infusion mass spectroscopy (MS) revealed the presence of multiple size species for each phospholipid class detected by $^{31}$P-NMR, indicating heterogeneity in the composition of fatty acid esters for each head group (Tables S1–S6). MS analysis also revealed the presence of trace amounts of PS not detected by $^{31}$P-NMR and confirmed the absence of sphingolipids and PG.

To see which of the components present in the purified preparation is responsible for cofactor activity, we tested commercial preparations of each phospholipid identified by $^{31}$P-

**Table 1. $^{31}$P-NMR analysis of purified cofactor preparation**

| Compound | Concentration, mM | Nominal MW | Amount % |
|---|---|---|---|
| PI | 0.301 | 884 | 4.3 |
| Lyso PC | 0.603 | 519 | 5.0 |
| Lyso PE | 0.413 | 495 | 3.3 |
| PC | 2.865 | 777 | 36.0 |
| PE | 4.122 | 770 | 51.3 |

MW, molecular weight.

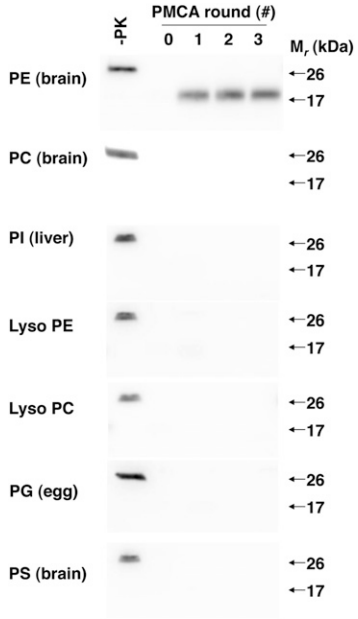


**Fig. 2.** Effect of various phospholipids on prion formation. Western blots showing three-round sPMCA reactions using recPrP substrate and seeded with recPrP$^{Sc}$ template, supplemented with various commercial preparations of purified and synthetic phospholipids at 2.5 mM final concentration, as indicated.

NMR. Our results showed that brain-derived PE robustly facilitates sPMCA reactions, whereas brain-derived PC, liver-derived PI, lyso PE, and lyso PC do not (Fig. 2). Previous work indicated that anionic phospholipids could influence PrP conformation and that the anionic phospholipid POPG, a synthetic form of PG, could facilitate the formation of recPrP$^{Sc}$ in the presence of liver RNA (5). Therefore, despite the lack of PG and PS in our purified preparation, we also tested these anionic phospholipids for their ability to facilitate sPMCA reactions in the absence of RNA. Unlike PE, neither PG (including POPG) nor PS displayed stimulatory activity in these assays (Fig. 2 and Fig. S3).

Brain-derived PE is a heterogeneous mixture (with respect to fatty acid composition) purified by HPLC from a mammalian source. We next tested whether synthetic C18 (plasmalogen) 18:1 PE (we chose this compound from among various commercially available synthetic PE phospholipids because it offered a combination of good chemical stability and detergent solubility) could support sPMCA propagation of recPrP$^{Sc}$ molecules. Our results (Fig. 3*A*) show that synthetic PE successfully propagates serial recPrP$^{Sc}$ propagation at concentrations ≥100 μM (Fig. S4). Moreover, the recPrP$^{Sc}$ molecules produced by serial propagation with synthetic PE are infectious, as judged by bioassay. Intracerebral inoculation of samples that had been serially propagated for 18 rounds (with a 1:10 seeding ratio in each round) into wild-type mice caused classical signs of scrapie in 100% of the inoculated animals after 381 ± 11 d, including shaking, ataxia, and impaired movement, whereas mice inoculated with control inocula remained asymptomatic (Table 2). The diagnosis of scrapie in symptomatic mice was confirmed by neuropathological analysis showing spongiform degeneration (Fig. 3*B*), Western blot analysis showing the presence of PrP$^{Sc}$ in the brains (Fig. 3*C*), and successful serial passage into normal C57BL hosts with a scrapie incubation period of 175 ± 4 d (Table 2).

Finally, we tested whether PE and other phospholipids are essential components for PrP$^{Sc}$ propagation in crude brain homogenates. We treated a crude homogenate of Prnp$^{0/0}$ brain with PLC, inactivated the enzyme by chelation, and tested the

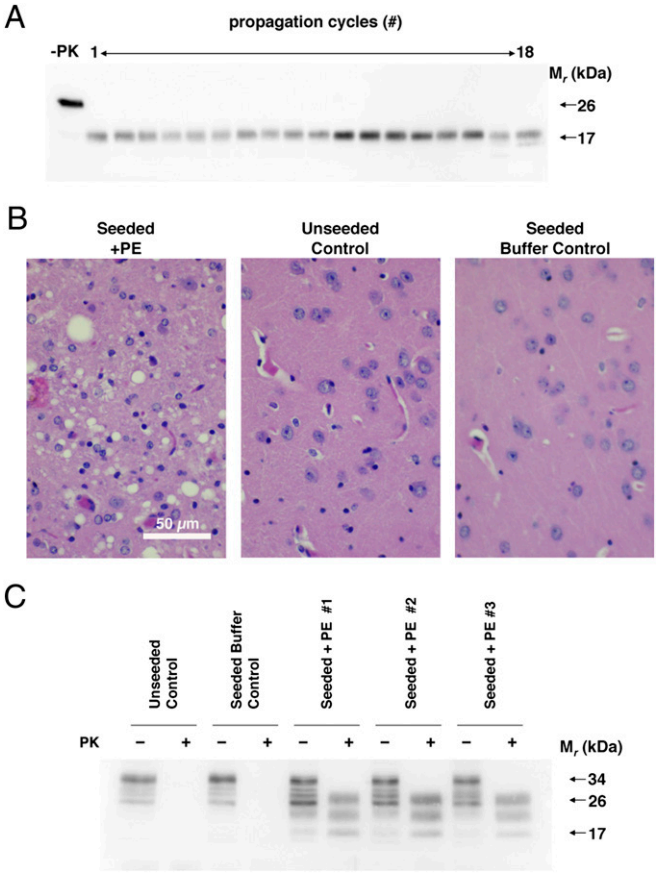


**Fig. 3.** Generation of infectious prions with synthetic plasmalogen PE. (*A*) Western blot showing 18-round sPMCA reactions containing only recPrP and 1 mM synthetic plasmalogen PE seeded with recPrP$^{Sc}$ in the first round. (*B*) Hematoxylin and eosin stained microscopic section of hypothalamus from mice inoculated with final sPMCA product of experiment shown in *A*, unseeded substrate mixture not subjected to sPMCA, or seeded mock sPMCA reactions in control buffer, as indicated. (*C*) Western blot to detect proteinase K-resistant PrP$^{Sc}$ in brain homogenates of inoculated mice. Samples from three different animals inoculated with the same preparation of recPrP$^{Sc}$ (seeded + PE) are shown.

treated homogenate for its ability to reconstitute both hamster and mouse PrP$^{Sc}$ propagation. Our results show that PLC treatment abolishes the ability of crude brain homogenate to facilitate PrP$^{Sc}$ propagation for both species, suggesting that one or more phospholipids are essential cofactors for PrP$^{Sc}$ formation in vitro (Fig. 4).

## Discussion

In this manuscript, we have identified PE as an endogenous cofactor that by itself can facilitate prion propagation using PrP molecules from multiple animal species. Previous studies have shown that the anionic phospholipids PS and POPG promote PrP conformational change (8, 9) and that POPG can facilitate the formation of infectious mouse prions in the presence of RNA molecules (5). Surprisingly, our reconstitution studies indicate that anionic phospholipids (PI, PS, and PG) are unable to facilitate prion propagation in the absence of RNA molecules (the absence of RNA molecules in our experiments was assured by the use of pure recPrP and synthetic phospholipid substrates, and the inclusion of a nuclease digestion step in the cofactor purification protocol). The ability of PE to serve as a solitary cofactor for prion propagation in vitro suggests that it may interact with PrP in a different way than anionic phospholipids.

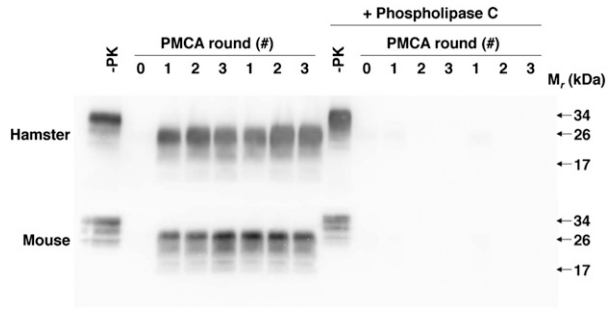


**Fig. 4.** Effect of PLC treatment on $PrP^{Sc}$ formation in brain homogenates. PLC-treated and control crude $Prnp^{0/0}$ brain homogenates were treated as described in *Materials and Methods* and used to reconstitute purified native hamster or mouse $PrP^{C}$ substrate in duplicate three-round sPMCA reactions seeded with either hamster Sc237 or mouse Me7 prions, as indicated. The control samples were not exposed to PLC, but otherwise mock processed together with the experimental samples (i.e., incubated with ZnCl2, dialyzed, and exposed to EDTA).

Consistent with this hypothesis, PE as a solitary cofactor facilitates a higher percentage conversion of recPrP substrate into protease-resistant PrP product than the combination of POPG plus RNA (Fig. S5). Also, unlike POPG, PE does not render recPrP insoluble before conversion to $PrP^{Sc}$ (Fig. S6).

Unlike RNA, which facilitates $PrP^{Sc}$ propagation in some animal species [including hamster (10, 11) and sheep (12)] but not others (including mouse and vole; ref. 7), PE facilitated propagation of $PrP^{Sc}$ from all four species tested, including hamster and deer. Moreover, our enzyme treatment/reconstitution studies indicate that one or more phospholipids play an essential role for the propagation of both hamster and mouse $PrP^{Sc}$ molecules in brain homogenates, whereas RNA is not required for mouse $PrP^{Sc}$ propagation. Taken together, these results suggest that PE is a highly promiscuous prion cofactor, whereas structural polyanions such as RNA may interact with species-specific PrP sequences, possibly to facilitate the formation of conformationally stable prion strains, as suggested by Gonzalez-Montalban et al. (11). The identification of structurally and functionally divergent cofactors in vitro suggests that different classes of endogenous molecules may serve to facilitate the propagation of different prion strains in vivo.

A prior study has shown that modest level of hamster prion infectivity can be propagated in sPMCA reactions by using purified recPrP alone, i.e., without deliberate addition of lipid or RNA molecules (13). However, the sPMCA conditions of Kim et al. (13) included the synthetic anionic detergent SDS, which may serve as an imperfect surrogate for naturally occurring cofactor molecules. The current study provides evidence that a naturally occurring molecule (PE) can stimulate the seeded conversion of recPrP into infectious prions.

Although we are unable to test by experimental manipulation whether PE or other phospholipids are required for in prion formation in vivo because membrane lipid levels are tightly regulated in cells, several lines of evidence suggest that brain-derived prions may contain essential polar lipids. (*i*) The specific infectivity of purified prion rods is increased 100-fold upon reconstitution into phospholipid liposomes (14). (*ii*) Prions are more easily inactivated by heat in the presence of fat rather than in water (15). (*iii*) Polar organic solvents that are able to extract polar lipids such 2-choroethanol inactivate prions, whereas nonpolar solvents such as hexane do not (16, 17). (*iv*) Variations in the strength of interaction between PrP and phospholipids correlate with differences in the thermostability of various prion strains (18, 19). (*v*) Radio inactivation studies suggest the presence of essential lipid molecules within infectious prions (20).

It is interesting to speculate that neuronal depletion of PE or another essential cofactor during the process prion formation could also mediate neurotoxicity, a process whose rate is apparently proportional to $PrP^{C}$ expression levels rather than infectious titer (21, 22). The rate of cofactor consumption during the asymptomatic phase of the disease would be proportional to the rate at which new $PrP^{C}$ substrate molecules were produced in infected neurons. In this scenario, it would not be necessary to invoke the induction of a neurotoxic PrP species to explain how $PrP^{C}$ to $PrP^{Sc}$ conversion causes cell death (22). It will be intriguing to see how cofactor molecules participate in the pathogenesis of human prion diseases, and other diseases in which protein misfolding appears to spread through the central nervous system (23).

## Materials and Methods

**Reagents.** The Sc237 and Me7 hamster prion strains used in this study were originally obtained from Stanley Prusiner (University of California, San Francisco, CA). Samples of $recPrP^{Sc}$ used as seed for sPMCA reactions with recPrP substrate was originally produced de novo by one of us (F.W.) as described (5) and subsequently propagated with purified cofactor or synthetic PE by N.R.D. and J.R.P. The pET-22b(+) expression plasmid (69744), Overnight Express Autoinduction System (71300-3), Bug Buster 10× plus Lysonase Kit (71370), and Ni-NTA His-Bind Superflow Resin (70691) were all purchased from EMD Chemicals. Sep-Pak Classic C18 cartridges were purchased from Waters Corporation. Micrococcal (S7) nuclease (107921) was purchased from Roche. Thermolysin (88303) and PLC, type V from *Bacillus cereus* (P4014) were both purchased from Sigma. Brain PE (840022P), PC (840053P), and PS (840032P); liver PI (840042P); Egg PG (841138P); synthetic plasmalogen PE (852758P), lyso PE (856705P), and lyso PC (855675P) were all purchased from Avanti Polar Lipids.

**$PrP^{C}$ Substrate and PrP27-30 Preparations.** Native mouse and hamster $PrP^{C}$ substrates were immunopurified from normal rodent brains as described (7). PrP27-30 preparations were prepared from scrapie-infected brains as described (4).

**Recombinant MoPrP Expression and Purification.** Amplified DNA sequences coding for mouse PrP 23–231 were ligated into the pET-22b(+) expression vector (EMD Chemicals) and sequences verified. The expression vector was then transformed into *E. coli* Rosetta Cells (EMD Chemicals). Cells were grown overnight in 1 L of LB (5 g of Yeast Extract, 10 g of Bacto Tryptone, and 10 g of NaCl) supplemented with the Overnight Express Auto-induction System (EMD Chemicals). The next day, the cells were centrifuged at 8,000 × *g* for 10 min and the supernatant was discarded. Pellets were resuspended in a solution of 1× Bug Buster and 10 μL of Lysonase (EMD Chemicals) containing EDTA-free Complete protease inhibitors (Roche). Cells were then incubated on ice and lysed by using intermittent sonication for 20 min. The lysate was centrifuged at 16,000 × *g* for 20 min and washed twice with 0.1×

**Table 2. Transmission of in vitro-generated recombinant prions to normal C57BL mice**

| Inoculum | $n/n_0$ | IP,* days |
|---|---|---|
| Eighteen-round sPMCA product of recPrP + plasmalogen PE mixture originally seeded with $recPrP^{Sc}$ | 8/8 | 381 ± 11 |
| Eighteen-round sPMCA product of buffer control mixture originally seeded with $recPrP^{Sc}$ | 0/4 | >530 |
| Unseeded recPrP + plasmalogen PE mixture | 0/4 | >530 |
| Serial passage of animals infected with plasmalogen PE-$recPrP^{Sc}$ prions | 8/8 | 175 ± 4 |

*Mean incubation period (IP) of scrapie sick animals ± SE. The samples of $recPrP^{Sc}$ used to seed sPMCA reactions was generated spontaneously as described (2).

Bug Buster. The resulting inclusion bodies were solubilized by using 8 M guanidine HCl and physical agitation, and insoluble material was removed by centrifugation at 8,000 × *g* for 15 min. PrP was then purified as described (5).

**Cofactor Preparation.** All centrifugation was done at 4 °C unless otherwise noted. A 10% (wt/vol) brain homogenate was made by processing 0.5 g of normal mouse brain in 4.5 mL of 20 mM Mops at pH 7.0 and 150 mM NaCl with a Potter homogenizer. Debris was removed by centrifugation for 30 s at 200 × *g*. The postnuclear supernatant was centrifuged for 30 min at 10,000 × *g*, and the resulting pellet was rehomogenized in 4.5 mL of 20 mM Mops at pH 7.0, 150 mM NaCl containing 3% (wt/vol) NOG (Anatrace), and incubated at room temperature for 30 min. Next, the homogenate was centrifuged at 100,000 × *g* for 60 min. The resulting supernatant was adjusted to 2 mM $CaCl_2$ and 150 U/mL S7 nuclease (Roche) and incubated at 37 °C for 30 min by using an end-over-end rotator. Thermolysin (Sigma) was added at a final concentration of 25 μg /mL, and the sample was incubated at 70 °C for 60 min with intermittent mixing. Next, the sample was cooled on ice, adjusted to 5 mM EDTA, and centrifuged for 1 h at 100,000 × *g*. The supernatant was then placed in cellulose ester dialysis tubing with a 20,000 molecular weight cutoff (Spectrum Laboratories) and dialyzed at 4 °C against water. After dialysis, the sample centrifuged for 3 h at 100,000 × *g*. The supernatant was discarded, and the pellet was resuspended in 1 mL of deionized water (one-fifth of the original homogenate volume).

**Saponification of Cofactor.** Two hundred microliters of purified cofactor preparation was adjusted to 3 M NaOH by the addition of 62.5 μL of 10 M NaOH and incubated for 1 h at 95 °C. After saponification, the sample was neutralized by adding 62.5 μL of concentrated HCl and buffered by the addition of 175 μL of 1 M Tris at pH 7.0. The resulting 500-μL sample was dialyzed overnight against 2 L of deionized water by using a 3,500 MWC Slide-A-Lyzer dialysis cassette (Thermo-Fisher Scientific). After dialysis, the sample was centrifuged for 1 h at 100,000 × *g*, and the supernatant was discarded. The saponified cofactor pellet was then resuspended in 200 μL of deionized water.

**PLC Treatment.** For PLC treatment of purified cofactor, the final dialysis step of the cofactor preparation protocol was omitted to maintain cofactor solubility. Twenty microliters of a buffer containing 3.2 M ammonium sulfate and 50 mM Tris at pH 7.5, either alone or containing 2,000–3,000 U/mL PLC (Sigma), was added to 475 μL of cofactor containing ~7 mM $ZnCl_2$. Samples were then incubated at 37 °C for 1 h, shaking at 600 rpm in an Eppendorf Thermomixer (Fisher Scientific). After incubation, samples were transferred to 10,000 MWC Slide-A-Lyzer dialysis cassettes (Thermo-Fisher Scientific), and dialyzed against 4 L of deionized water for 72 h, changing the water every 24 h. Samples were then removed from cassettes and centrifuged for 1 h at 100,000 × *g*. The resulting pellets were then resuspended in 400 μL of deionized water.

For PLC treatment of brain homogenate, 1.25 mL of 10% (wt/vol) $Prnp^{0/0}$ mouse brain homogenate was adjusted to 0.25% Triton X-100 and 4 mM $ZnCl_2$. Sixty microliters of a buffer containing 3.2 M ammonium sulfate and 50 mM Tris at pH 7.5, either alone or containing 2,000–3,000 U/mL PLC (Sigma), was added and samples were incubated at 37 °C for 1 h, shaking at 600 rpm in an Eppendorf Thermomixer (Fisher Scientific). Next, 0.5 mM EDTA at pH 7.5 was added to a final concentration of 6 mM. Samples were then transferred to 3,500 MWC Slide-A-Lyzer dialysis cassettes (Thermo-Fisher Scientific) and dialyzed against 4 L of PBS overnight. Treated homogenate was then removed from cassettes, and 0.5 mM EDTA at pH 7.5 was again added to a final concentration of 6 mM. The efficacy of inactivation by EDTA was confirmed by using Western blot to check that no signal reduction had occurred after incubation of $PrP^C$ with inactivated brain homogenate as a result of glycophosphatidylinositol-anchor hydrolysis (24).

**Silica Chromatography.** A 500-μL aliquot of cofactor was lyophilized and resuspended in 1 mL of chloroform. The sample was then applied to a Sep-Pak Classic C18 cartridge (Waters Corporation), preequilibrated with 10 mL of chloroform. Next, the cartridge was successively eluted with 10 mL of chloroform, 15 mL of acetone:methanol (9:1), and 10 mL of methanol. All three eluates were collected (separately), dried down under nitrogen gas, and resuspended in 1 mL of water.

**Serial Protein Misfolding Cyclic Amplification.** Reconstituted sPMCA reactions using brain-derived $PrP^C$ were conducted as reported by using either Sc237 or Me7 PrP27-30 molecules as seed for the Day 1 reaction (7). Reactions in subsequent rounds were seeded with 10 μL of product from the prior round. Reactions using recombinant MoPrP were performed by using a similar technique with slight modification. Briefly, sonication pulses were 15 s, and 100-μL reactions contained 6 μg /mL recombinant MoPrP, 20 mM Tris at pH 7.5, 135 mM NaCl, 5 mM EDTA at pH 7.5, and 0.15% Triton X-100 supplemented with cofactor where indicated. In all experiments using recPrP substrate, Day 1 reactions were seeded with 10 μL of $recPrP^{Sc}$ originally produced de novo as described (5) and subsequently propagated with purified cofactor or synthetic PE (this material was chosen as seed to match the recPrP substrate with a $recPrP^{Sc}$ seed). Lipids were tested at three final concentrations, 2.5, 1.0, and 0.5 mM. Each lipid was resuspended from powder with 0–1.5% Triton X-100, depending on solubility. PS was dissolved in water; plasmalogen PE was dissolved in 0.04% Triton; PE, PI, Lyso-PE and Lyso-PC were dissolved in 0.2% Triton; PC was dissolved in 0.5% Triton X-100, and PG was resuspended in 1.5% Triton X-100.

**$PrP^{Sc}$ Detection.** To detect $PrP^{Sc}$ molecules, samples containing hamster prions were digested with 50 μg/mL proteinase K (PK) for 1 h at 37 °C, and samples containing mouse prions (including all recPrP samples) were digested with 25 μg/mL PK for 30 min at 37 °C. All samples were processed for SDS/PAGE and Western blotting as described (25), substituting Towbin transfer buffer (26) and using mAb 6D11 as the primary antibody.

**Scrapie Inoculation and Diagnosis.** Intracerebral inoculation and diagnosis of prion disease were performed as described (25). PMCA mixtures and products were diluted 1:10 into PBS plus 1% BSA before inoculation. The inoculum volume used was 30 μL.

**Neuropathology.** Brains were removed rapidly at the time of sacrifice by using new, sterile-packaged dissection instruments and disposable surfaces to avoid cross-contamination. They were immersion fixed in 10% buffered formalin for 2–30 d, cut into ~3-mm-thick saggital sections, and placed in a tissue-processing cassette. Cassettes were treated with 88% formic acid for 1 h, and then stored in PBS. The tissue was processed for paraffin embedding, and representative slides were stained with hematoxylin and eosin (H&E). Immunohistochemistry was performed on deparrafinized slides by using 2 μg/mL 27/33 anti-PrP mAb for 30 min at room temperature after citrate antigen retrieval and a Biocare Mouse on Mouse development kit.

**Sample Preparation for NMR ($^{31}$P-NMR) and Flow Infusion Mass Spectrometry (MS).** A sample containing 5.1 mg of cofactor preparation was dissolved in 1 mL of HPLC grade chloroform. For MS analysis, 200 μL was removed for dilution into a 10-mL volumetric flask with 10 mM $NH_4OAc$, 0.01% concentration $NH_4OH$ in methanol. The final concentration of cofactor preparation was equivalent to 102 μg/mL. The remaining 4.8 mg was dried under nitrogen and dissolved in 1.0 mL of detergent solution for $^{31}$P-NMR analysis. Both analyses were performed by Avanti Polar Lipids.

**$^{31}$P-NMR Analysis.** The detergent sample solution was assayed on a Bruker Advance 400 MHz instrument. Phosphorus response was calibrated with a known standard of 18:1 PC for measurement of phospholipids in the sample. The sample received 1,024 scans with nuclei relaxation time of 1 s for optimum sensitivity.

**Flow Infusion Mass Spectrometry.** The 102 μg/mL solution was directly infused into the interface of an API 4000 Qtrap mass spectrometer at 20 μL/min. The solution was scanned under MS/MS techniques to selectively detect major phospholipid and sphingoid base lipids. Table S7 outlines the MS/MS parameters used for each lipid class.

The possible identity of the mass ions detected for each lipid class was searched against a lipid mass spectral database at www.lipidmaps.org. The counts per second (cps) intensities of each ion peak within a class specific mass spectrum were nomalized to the total. The nominal molecular weight calculated was used to convert millimolars of phosphorus to phospholipid in the $^{31}$P-NMR results. The search engine results were interpreted to provide a proposed identity of the compounds according to the number of carbons in the fatty acyl chains of the phospholipid structure with corresponding number of double bonds. The identity was based on a general assumption related to sample origin. Phospholipids with odd-numbered fatty acyl chains are uncommon in mammalian systems. In the event of odd and even chain options of identity, chain compounds were chosen. Finally, the cps abundances of the chosen compounds were normalized against the total to provide a relative abundance of compounds within the lipid class.

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