

# Requirements for Mutant and Wild-Type Prion Protein Misfolding In Vitro

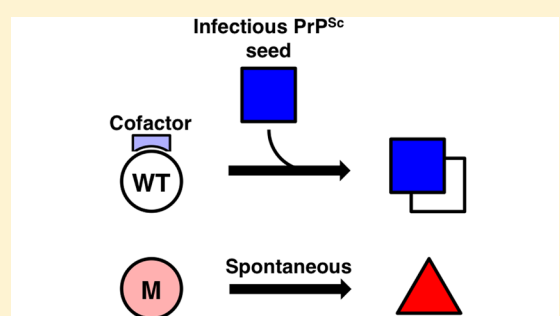
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## S Supporting Information

**ABSTRACT:** Misfolding of the prion protein (PrP) plays a central role in the pathogenesis of infectious, sporadic, and inherited prion diseases. Here we use a chemically defined prion propagation system to study misfolding of the pathogenic PrP mutant D177N in vitro. This mutation causes PrP to misfold spontaneously in the absence of cofactor molecules in a process dependent on time, temperature, pH, and intermittent sonication. Spontaneously misfolded mutant PrP is able to template its unique conformation onto wild-type PrP substrate in a process that requires a phospholipid activity distinct from that required for the propagation of infectious prions. Similar results were obtained with a second pathogenic PrP mutant, E199K, but not with the polymorphic substitution M128V. Moreover, wild-type PrP inhibits mutant PrP misfolding in a dose-dependent manner, and cofactor molecules can antagonize this effect. These studies suggest that interactions between mutant PrP, wild-type PrP, and other cellular factors may control the rate of PrP misfolding in inherited prion diseases.



Wild Type versus Mutant Prion Protein Misfolding

Prion diseases are unique in their occurrence via infectious, sporadic, and genetic etiologies. In the infectious and sporadic forms of these diseases, the normal, host-encoded prion protein (PrP<sup>C</sup>) undergoes conformational change into a self-propagating, misfolded conformer termed PrP<sup>Sc</sup>, an essential component of infectious prions.<sup>1</sup> Misfolding of PrP also plays a central pathogenic role in inherited forms of prion disease, but it is unknown whether the process by which PrP mutations promote the development of self-propagating conformations is mechanistically related to the process of infectious prion formation from wild-type (WT) PrP.

Our understanding of the templated misfolding of PrP that underlies prion propagation has been significantly advanced by the development of in vitro prion conversion assays,<sup>2–4</sup> which provide a tool for defining the biochemical components and interactions required for prion formation. Recently, Deleault et al. reported an in vitro prion conversion system capable of producing high titer mouse prions using only recombinant PrP and a single endogenous cofactor molecule, the phospholipid phosphatidylethanolamine (PE).<sup>5</sup> This chemically defined, minimal system was used to show that cofactor molecules play an essential role in maintaining the infectious properties of prions.<sup>6</sup> It is not known whether cofactor molecules play similarly important roles in the PrP misfolding associated with genetic prion diseases.

Fatal familial insomnia (FFI) and familial Creutzfeldt-Jakob disease (fCJD) are genetic prion diseases of humans caused by the D178N and E200K mutations in PrP, respectively (homologous to D177N and E199K in mouse (Mo)PrP).<sup>7,8</sup> Jackson et al. recently modeled both of these inherited prion diseases using knock-in transgenic mice<sup>9,10</sup> and observed the production of spontaneous clinical disease that is transmissible to animals which lack pathogenic PrP mutations. These results suggest that the D177N and E199K mutations have a profound effect on PrP misfolding.

We saw an opportunity to investigate in vitro the biochemical events and functional interactions that influence PrP misfolding in genetic prion disease by using a chemically defined, minimal system that has been previously shown to be capable of propagating infectious prions. Our results show that, unexpectedly, cofactor and WT PrP molecules play important roles in the propagation of mutant PrP conformations.

## EXPERIMENTAL PROCEDURES

**Recombinant PrP Expression and Purification.** Recombinant MoPrP 23–230, hereafter referred to as WT recPrP, was expressed and purified by reversed-phase HPLC as

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described elsewhere.<sup>11</sup> To generate recombinant D177N MoPrP 23–230, hereafter referred to as D177N recPrP, an expression construct was produced using the GeneTailor site-directed mutagenesis system (Invitrogen, Grand Island, NY), with the pET-22b(+) vector (EMD Millipore, Billerica, MA) containing the WT recPrP sequence as template and the following mutagenic primers: 5'-AACAACTTCGTGCA-CAACTGCGTCAATATC-3', 5'-GTGCACGAAGTTGTTCTGGTTAGAGTAC-3'. DNA sequencing of the entire PrP coding region confirmed the sequence of the D177N recPrP expression vector, and D177N recPrP was expressed and purified as described for WT recPrP.<sup>11</sup> E199K MoPrP 23–230 (E199K recPrP) and M128V MoPrP 23–230 (M128V recPrP) were generated as described above for D177N recPrP using the following mutagenic primer pairs:

5'-CCAAGGGGAGAACTTCACCAAGACCGATGTGAA-3', 5'-GGTGAAGTTCTCCCCCTTGGTGGTGGTGGTGA-3' and 5'-TGGGGGGCCCTAGGTGGCTACGTGCTGGGGAGTGCCATG-3', 5'-GTAGCCACCTAGGCC-CCCCACCACGGCCCTGCAG-3', respectively.

**Circular Dichroism Spectroscopy.** D177N recPrP was resuspended to 0.18 mg/mL in water, and a circular dichroism (CD) spectrum was collected on a Jasco J-715 spectropolarimeter, reading wavelengths from 190 to 280 nm at a rate of 20 nm/min, and as otherwise described previously.<sup>12</sup>

**Brain-Derived Cofactor Preparation.** Brain-derived cofactor was prepared as described previously<sup>5</sup> using normal rat brain as the tissue source and with the following modifications: after thermolysin treatment and centrifugation, the supernatant was frozen at -70 °C and thawed before placing in a 3500 molecular weight cutoff Slide-A-Lyzer cassette (Thermo Fisher Scientific, Rockford, IL) for dialysis against water at 4 °C.

**Serial PMCA with Recombinant PrP Substrates.** Serial protein misfolding cyclic amplification (sPMCA) was performed as described previously.<sup>5</sup> Unless otherwise indicated, reactions contained 6 µg/mL recPrP, 20 mM Tris (pH 7.5), 135 mM NaCl, 5 mM EDTA (pH 7.5), 0.15% (v/v) Triton X-100 and were supplemented with either brain-derived cofactor, pure phospholipids, or water. Reactions were seeded with 1/10th volume of converted PMCA product or unconverted substrate cocktail and sonicated with 15-s pulses every 30 min for 24 h at 37 °C. 1/10th volume of the reaction was then used to seed fresh substrate cocktail, and the 24 h sonication program was repeated. All reactions were sonicated in microplate horns using a Misonix S-4000 power supply (Qsonica, Newtown, CT) set to an amplitude of 50–70. To prevent cross-contamination, sample tubes were sealed with Parafilm (Bemis Flexible Packaging, Oshkosh, WI), and the sonicator horn was soaked in 100% bleach between experiments.

**Phospholipase Treatment of Brain-Derived Cofactor.** Prior to the dialysis step, brain-derived cofactor was supplemented with ZnCl<sub>2</sub> to overwhelm residual EDTA and then treated with phospholipase C (PLC) from *B. cereus* (Sigma-Aldrich, St. Louis, MO, product P6621). Briefly, PLC was resuspended in water to 1 mg/mL protein, and an aliquot was inactivated by incubation at 95 °C for 30 min. The ZnCl<sub>2</sub>-supplemented cofactor was treated with 0.1 U/µL PLC, or the equivalent amount of heat-inactivated enzyme, for 1 h at 37 °C. Treated cofactor was then dialyzed against water as described above.

**Phospholipid Resuspension.** Purified phospholipids were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Synthetic phosphatidylethanolamine (Product 852758P) and brain-derived phosphatidylcholine (Product 840053P) stocks were resuspended to 10 mM from powder in 0.04% (v/v) Triton X-100 by sonication at 4 °C under nitrogen gas and in the dark. Resuspended lipids were added to sPMCA reaction buffer and incubated at 37 °C for 5 min to improve solubility prior to the assembly of sPMCA substrate cocktail.

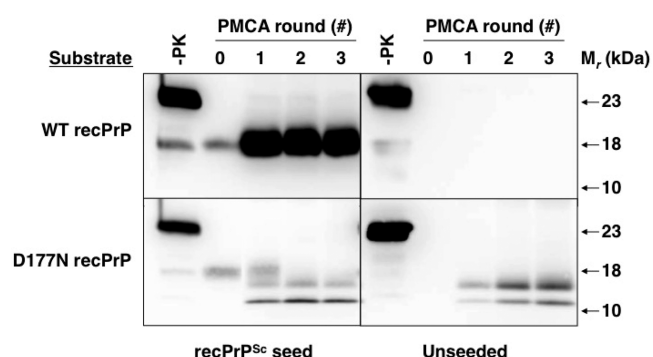
**PrP<sup>res</sup> Detection.** To detect misfolded, protease-resistant PrP, termed PrP<sup>res</sup>, the products of PMCA reactions containing recombinant PrP substrates were digested with proteinase K (PK) and analyzed by SDS-PAGE and Western blotting as previously described<sup>6</sup> using anti-PrP mAb 27/33 as the primary antibody unless otherwise specified.

## RESULTS

### Spontaneous Generation of D177N recPrP<sup>res</sup> in Vitro.

Previously, Deleault et al. reported the generation of high titer infectious prions in vitro using only murine WT recPrP, hereafter referred to as WT recPrP, and a purified lipid cofactor.<sup>5</sup> This defined minimal system provides a unique opportunity to study the mechanism by which familial prion disease mutations promote PrP misfolding.

To test whether a pathogenic mutation alters the seed and/or cofactor requirements for PrP misfolding, we generated and purified murine recPrP containing the D177N mutation, which causes FFI. CD spectroscopy was performed to confirm that this mutant PrP molecule adopts an  $\alpha$ -helical fold (Figure S1, Supporting Information). D177N recPrP was then used as a substrate for serial protein misfolding cyclic amplification (sPMCA) reactions supplemented with brain-derived cofactor, in the presence or absence of seeding with recPrP<sup>Sc</sup> (Figure 1).

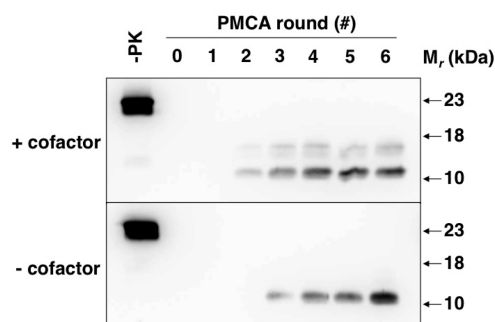


**Figure 1.** Effect of seeding on recPrP<sup>Sc</sup> and D177N recPrP<sup>res</sup> formation and propagation in vitro. Western blots showing three rounds of recPrP<sup>Sc</sup>-seeded or unseeded sPMCA reactions using D177N or WT recPrP as the substrate. All reactions are supplemented with brain-derived cofactor and seeded as indicated. Experiment was performed two times with similar results.

In this manuscript we use recPrP<sup>Sc</sup> to refer specifically to the infectious WT recPrP conformation that was previously reported by Deleault et al.<sup>6</sup> In contrast to WT recPrP, which remains sensitive to PK when sPMCA reactions are unseeded (Figure 1, top right panel), D177N recPrP spontaneously adopts a PK-resistant conformation, which we refer to as D177N recPrP<sup>res</sup>, in the absence of a PrP<sup>Sc</sup> seed within 1–3 propagation rounds (Figure 1, bottom right panel). This spontaneous conversion of D177N recPrP occurs most

efficiently at physiological temperature and pH, and requires intermittent sonication (Figure S2, Supporting Information). Moreover, the D177N recPrP<sup>res</sup> produced differs phenotypically from recPrP<sup>Sc</sup> when subjected to PK digestion: D177N recPrP<sup>res</sup> appears as a weak doublet on Western blot, whereas PK-digested recPrP<sup>Sc</sup> appears as a single strong band at approximately 18 kDa, as previously reported.<sup>6</sup> In some instances, PK-digested D177N recPrP<sup>res</sup> appears to contain more than two bands, although in these cases subtle splitting of bands in undigested samples is also observed, suggesting that this is an artifact of the SDS-PAGE or Western blotting process.

**Spontaneous Generation of D177N recPrP<sup>res</sup> Does Not Require a Cofactor.** Since D177N recPrP adopts a PK-resistant conformation in the absence of PrP<sup>Sc</sup> seed, we wondered whether it might also spontaneously misfold in the absence of cofactor molecules. To test this, we carried out unseeded, D177N recPrP sPMCA with or without brain-derived cofactor supplementation (Figure 2). Even in the



**Figure 2.** Role of cofactor in the spontaneous formation and propagation of D177N recPrP<sup>res</sup>. Western blots showing six rounds of unseeded sPMCA reactions using D177N recPrP as the substrate in the presence or absence of brain-derived cofactor. Experiment was performed four times with similar results.

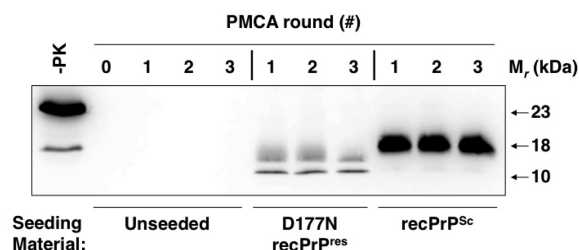
absence of cofactor, D177N recPrP spontaneously adopts a PK-resistant conformation (Figure 2, bottom panel). Interestingly, the upper band of the D177N recPrP<sup>res</sup> doublet appears more sensitive to protease digestion in the absence of cofactor molecules (Figure 2, top versus bottom panels).

We also examined the cofactor dependence of two other PrP sequences: a second pathogenic substitution, E199K,<sup>8</sup> and a polymorphic variant, M128V.<sup>13</sup> Our results show that, like D177N recPrP, E199K recPrP forms a weakly PK-resistant doublet in the presence or absence of cofactor supplementation (Figure S3A, top panel, Supporting Information). This PK-resistant conformer, which we refer to as E199K recPrP<sup>res</sup>, also forms spontaneously in the absence of seeding with recPrP<sup>Sc</sup> (Figure S3B). In contrast to both D177N and E199K recPrP, M128V recPrP forms recPrP<sup>Sc</sup> in a cofactor-dependent manner (Figure S3A, bottom panel), as is seen for WT recPrP.<sup>6</sup>

**Epitope Mapping of D177N recPrP<sup>res</sup> after PK Digestion.** To characterize the PK-resistant fragments generated from D177N recPrP<sup>res</sup>, we performed epitope mapping using anti-PrP monoclonal antibodies (mAbs) (Figure S4, Supporting Information). The PK-resistant doublet detected throughout this study by mAb 27/33 (epitope predicted to reside between residues 134 and 144, see Figure S4) is comprised of two C-terminal fragments with relative mobilities ( $M_r$ ) of approximately 16 and 12 kDa (Figure S4, left and middle panels). The larger, 16 kDa fragment also contains

the epitopes for mAbs 6D11 (residues 97–100) and R2 (residues 224–230), indicating that it includes all residues C-terminal to lysine 100 (Figure S4, left, middle, and right panels).<sup>14,15</sup>

**D177N recPrP<sup>res</sup> Can Drive the Misfolding of WT recPrP.** Individuals who carry an FFI mutant allele almost always also carry a normal, WT allele of *PRNP*, the gene encoding PrP. Therefore, we wondered whether spontaneously generated D177N recPrP<sup>res</sup> might be able to function as a seed to drive the misfolding of WT recPrP. To test this, we used D177N recPrP<sup>res</sup> as a seed in sPMCA reactions containing WT recPrP and supplemented with brain-derived cofactor (Figure 3). D177N recPrP<sup>res</sup> was able to convert WT recPrP into a PK-



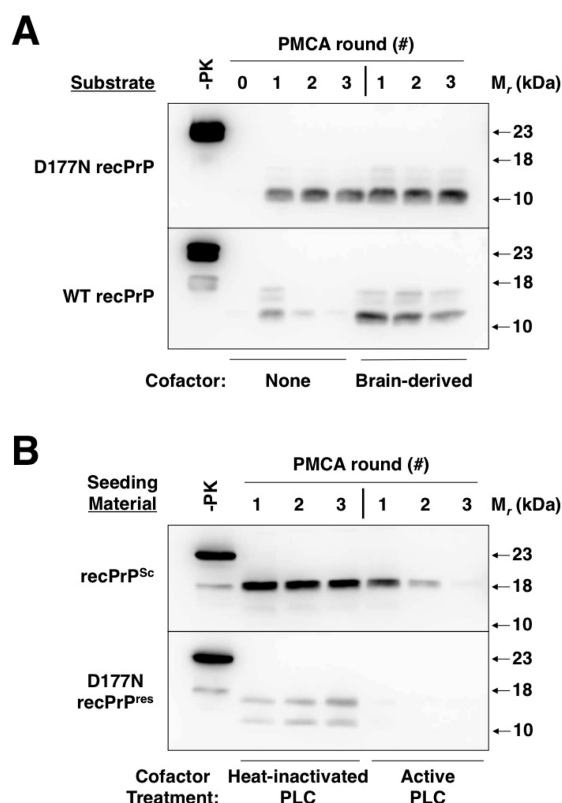
**Figure 3.** Propagation of D177N recPrP<sup>res</sup> and infectious recPrP<sup>Sc</sup> conformers in WT recPrP substrate. Western blot showing three-round sPMCA reactions using WT recPrP as the substrate and seeded as indicated. All reactions are supplemented with brain-derived cofactor. Experiment was performed three times with similar results.

resistant form that is phenotypically similar the original D177N recPrP<sup>res</sup> seed in its protease digestion pattern (Figure 3, middle sample group). This D177N-derived recPrP<sup>res</sup> propagating in WT recPrP substrate is distinct from the 18 kDa PK-resistant core formed when that same WT substrate is seeded with infectious recPrP<sup>Sc</sup> (Figure 3, right sample group) and will be referred to as WT recPrP<sup>res</sup> (D177N-seeded). Thus, D177N recPrP<sup>res</sup> is able to template its unique conformation onto WT recPrP. We refer to this adaptation of D177N recPrP<sup>res</sup> to propagation in WT recPrP substrate as cross-propagation.

**D177N recPrP<sup>res</sup> Cross-Propagation Requires a Phospholipid Cofactor.** Since D177N recPrP<sup>res</sup> maintained its conformation when cross-propagated into WT recPrP substrate, we wondered whether the ability of this conformer to propagate in the absence of cofactors was also maintained after adaptation to WT recPrP. To test this, we used D177N recPrP<sup>res</sup> to seed sPMCA reactions containing either D177N or WT recPrP substrate with or without brain-derived cofactor (Figure 4A). We found that in order for D177N recPrP<sup>res</sup> to cross-propagate efficiently into WT recPrP substrate, brain-derived cofactor was required (Figure 4A, bottom panel). Brain-derived cofactor was also required for cross-propagation of E199K recPrP<sup>res</sup> into WT recPrP substrate (Figure S3C).

To date, the only cofactor-dependent PrP conformations that have been reported in sPMCA experiments are infectious PrP<sup>Sc</sup>.<sup>4,5,16</sup> Recently, we showed that the component of our brain-derived cofactor that is active in the propagation of infectious recPrP<sup>Sc</sup> is a single phospholipid species, phosphatidylethanolamine (PE). We, therefore, wondered whether one or more phospholipids might also be active in the cross-propagation of D177N recPrP<sup>res</sup> into WT recPrP substrate. We tested this hypothesis by treating brain-derived cofactor with phospholipase C (PLC), which hydrolyzes the phosphate bonds of most phospholipid species, including glycerophos-

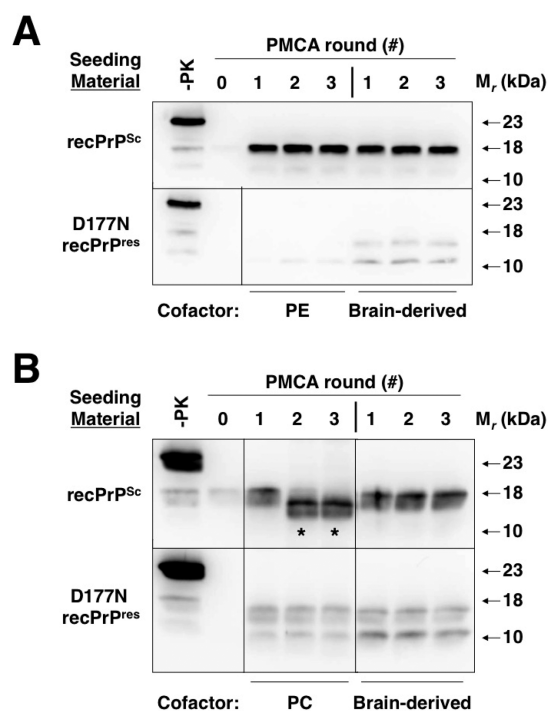




**Figure 4.** Role of cofactor in cross-propagation of D177N recPrP<sup>res</sup> into WT recPrP. (A) Effect of brain-derived cofactor on D177N recPrP<sup>res</sup> cross-propagation. Western blot showing three-round sPMCA reactions using D177N or WT recPrP as the substrate, either with or without the addition of brain-derived cofactor, as indicated. All reactions are seeded with spontaneously misfolded D177N recPrP<sup>res</sup>. Experiment was performed three times with similar results. (B) Effect of phospholipase C on D177N recPrP<sup>res</sup> cross-propagation. Western blot showing three-round sPMCA reactions supplemented with phospholipase C- or mock-treated cofactor. All reactions use WT recPrP as the substrate and are seeded as indicated.

pholipids, ceramide, and plasmalogen phospholipids, and cardiolipin. PLC-treated cofactor is unable to facilitate infectious recPrP<sup>Sc</sup> propagation (Figure 4B, top panel), as previously reported.<sup>5</sup> Cross-propagation of D177N recPrP<sup>res</sup> is also inhibited by treatment of cofactor with PLC (Figure 4B, bottom panel), indicating that one or more phospholipids are active in the conversion of WT recPrP to the D177N recPrP<sup>res</sup> conformation.

**WT recPrP<sup>res</sup> (D177N-Seeded) Utilizes a Different Phospholipid Cofactor than Infectious recPrP<sup>Sc</sup>.** Next, with two different, phospholipid-dependent conformations of WT recPrP (WT recPrP<sup>res</sup> (D177N-seeded) and recPrP<sup>Sc</sup>) in hand, we wondered whether these distinct conformations both utilize PE as a cofactor or whether WT recPrP<sup>res</sup> (D177N-seeded) might have a different phospholipid specificity than recPrP<sup>Sc</sup>. To test whether both recPrP conformations are dependent on the same cofactor activity, we used purified PE to reconstitute sPMCA reactions seeded with either recPrP<sup>Sc</sup> or D177N recPrP<sup>res</sup> (Figure 5A). Our results show that purified PE is unable to reconstitute the cross-propagation of D177N recPrP<sup>res</sup> into WT recPrP substrate (Figure 5A, bottom panel), but fully reconstitutes the propagation of infectious recPrP<sup>Sc</sup> (Figure 5A, top panel), as previously reported.<sup>5</sup> This result indicates that WT recPrP<sup>res</sup> (D177N-seeded) has a different

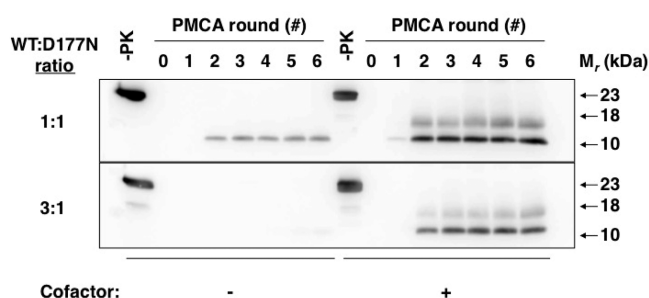


**Figure 5.** Attempted reconstitution of D177N recPrP<sup>res</sup> cross-propagation with purified phospholipids. Western blots of three-round sPMCA reactions using WT recPrP as the substrate and supplemented with purified phospholipid or brain-derived cofactor. (A) Attempted reconstitution of D177N recPrP<sup>res</sup> cross-propagation with phosphatidylethanolamine (PE). Experiment was performed two times with similar results. (B) Reconstitution of D177N recPrP<sup>res</sup> cross-propagation with phosphatidylcholine (PC). Lanes marked with an asterisk (\*) indicate the appearance of the protein-only PrP<sup>Sc</sup> conformer due to insufficient cofactor activity, as described previously.<sup>6</sup> Experiment was performed two times with similar results.

phospholipid cofactor specificity than recPrP<sup>Sc</sup>. Importantly, this distinct phospholipid cofactor specificity arises only after the D177N recPrP<sup>res</sup> conformation is cross-propagated into WT recPrP substrate, which implies that it is conformation, and not protein primary sequence, that is determining cofactor specificity.

While PE is the major phospholipid component (~51%) of our brain-derived cofactor preparation, phosphatidylcholine (PC) is also a significant contributor, accounting for approximately 36% of the phospholipid content.<sup>5</sup> We, therefore, tested whether purified PC could reconstitute cross-propagation of D177N recPrP<sup>res</sup> into WT recPrP substrate (Figure 5B). As expected, sPMCA reactions seeded with infectious recPrP<sup>Sc</sup> and containing purified PC as the sole cofactor do not sustain recPrP<sup>Sc</sup> propagation (Figure 5B, top panel, left sample group). By the second day of amplification, there is a shift to the lower molecular weight, protein-only PrP<sup>Sc</sup> conformation, indicating insufficient cofactor activity, as reported previously.<sup>6</sup> In contrast, when sPMCA reactions containing PC as the sole cofactor are seeded with D177N recPrP<sup>res</sup>, propagation of a weakly PK-resistant doublet is maintained (Figure 5B, bottom panel, left sample group). However, the digestion pattern of this doublet differs subtly from that produced during propagation with the brain-derived cofactor, with both PK-resistant bands running at a slightly higher  $M_r$ .

**Cofactor Molecules Relieve Heteroallelic Inhibition of D177N Misfolding.** Given that D177N recPrP, but not WT recPrP, spontaneously misfolds in sPMCA reactions, we wondered what effect a mixture of D177N and WT recPrP substrate might have on the de novo formation of recPrP<sup>res</sup>. Such a mixture simulates the misfolding environment encountered in the brains of individuals carrying the dominantly inherited FFI mutation. To test this, we performed unseeded sPMCA in which the ratio of D177N and WT recPrP substrate was varied while holding the concentration of D177N recPrP constant (Figure 6). When equimolar concentrations of



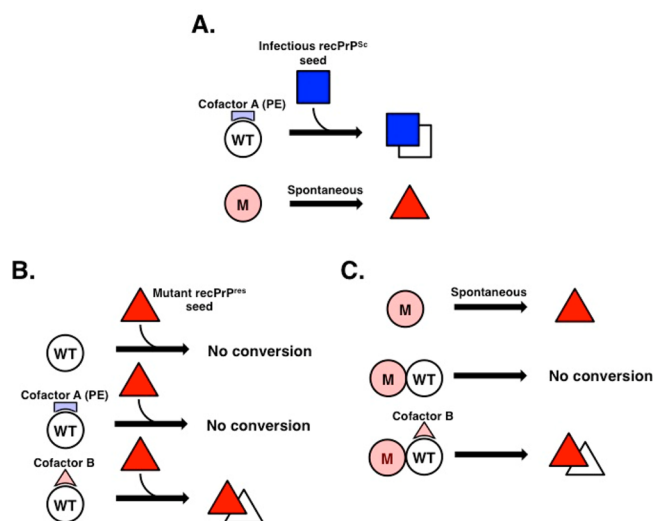
**Figure 6.** Effect of WT recPrP on the spontaneous misfolding of D177N recPrP. Western blots of six-round unseeded sPMCA reactions. All reactions contain 3  $\mu$ g/mL D177N recPrP combined with WT recPrP in the ratios indicated. Reactions were performed with or without the addition of brain-derived cofactor, as indicated. Experiment was performed two times with similar results.

D177N and WT recPrP were present, recPrP<sup>res</sup> is formed spontaneously (Figure 6, top panel). If, however, WT recPrP is present in excess, at a ratio to D177N of 3:1, the spontaneous production of recPrP<sup>res</sup> is abolished (Figure 6, bottom panel, left sample group). This inhibitory effect of the WT protein, which we have termed heteroallelic inhibition, is dramatically affected by the presence of cofactor molecules: in the presence of cofactor molecules, which facilitate the cross-propagation of D177N recPrP<sup>res</sup> (Figure 4), heteroallelic inhibition is relieved (Figure 6, bottom panel, right sample group).

## DISCUSSION

Previous studies have shown that highly infectious prions can be produced and propagated using WT PrP substrate in vitro, in a process requiring additional cofactor molecules.<sup>6</sup> However, it is unknown whether the misfolding of pathogenic mutant PrP molecules, a process initiated by the presence of a dominant mutant *PRNP* allele which does not necessarily lead to the production of infectious prions,<sup>17</sup> might proceed by a different pathway. In this study, we used a chemically defined system to study molecular interactions that might influence the misfolding of mutant (D177N and E199K) and WT PrP molecules. The major biochemical findings of this study are shown schematically in Figure 7.

**Mutant PrP Misfolds in the Absence of Seed or Cofactors.** Inherited prion diseases display a high degree of penetrance among patients harboring mutant *PRNP* alleles.<sup>18</sup> In contrast, the incidence of sporadic prion disease is extremely low,<sup>19</sup> and the development of infectious prion disease requires exposure to pre-existing infectious prions. These observations suggest that pathogenic mutations may allow mutant PrP molecules to misfold directly, bypassing the cofactor- and seed-facilitated mechanism of conformational change responsible for



**Figure 7.** Schematic representation of the major findings in this study. (A) WT and mutant recPrP molecules have different biochemical requirements for misfolding. (B) Cross-propagation of mutant recPrP conformers into WT recPrP substrate requires a distinct cofactor activity. (C) Heteroallelic inhibition of recPrP misfolding is relieved by cofactor activity. WT = WT recPrP, M = mutant recPrP, cofactor A (PE) = phosphatidylethanolamine (PE), cofactor B = non-PE, phospholipid-dependent cofactor activity. Circles represent normally folded,  $\alpha$ -helical PrP, squares represent the infectious recPrP<sup>Sc</sup> conformation, and triangles represent the mutant recPrP<sup>res</sup> conformation.

forming the infectious recPrP<sup>Sc</sup> conformation. We tested this hypothesis by using recombinant mutant D177N PrP molecules as substrates in sPMCA reactions. Our results, illustrated in Figure 7A, showed that D177N recPrP spontaneously misfolds into a protease-resistant conformer (D177N recPrP<sup>res</sup>) even in the absence of cofactor and infectious seed, providing a biochemical explanation for the high degree of penetrance associated with PrP mutations, while also highlighting mechanistic differences between the infectious and inherited prion diseases.

A comparison of global secondary structure by CD showed that, prior to sPMCA-induced misfolding, the conformation of D177N recPrP is similar to that of WT PrP. It is likely that the D177N mutation destabilizes a critical structural domain that normally participates in maintaining PrP conformation, thereby lowering the potential energy barrier to global misfolding. This interpretation is consistent with previous studies that have examined the effect of this pathogenic mutation on PrP's overall fold,<sup>20</sup> thermodynamic stability,<sup>21</sup> tendency to form amyloid,<sup>22,23</sup> and conformational dynamics.<sup>24–27</sup> In contrast, WT PrP may have a relatively stable structure, so cofactor molecules and infectious seed are needed to facilitate its conversion to PrP<sup>Sc</sup> by promoting the conformational change of domains that normally maintain the  $\alpha$ -helical structure of WT PrP.

Formation of D177N recPrP<sup>res</sup> in vitro is a time-, temperature-, and pH-dependent process requiring intermittent sonication. The sonication requirement suggests that, in addition to the intrinsic instability of mutant PrP molecules, a nucleation–polymerization process (in which partial disaggregation by sonication can accelerate the kinetics of misfolding exponentially by creating additional seeds<sup>28</sup>) might also play a role in promoting D177N misfolding. Consistent with this

interpretation, we observed that levels of D177N recPrP<sup>res</sup> often grew over the course of several sPMCA rounds.

**WT PrP Molecules Can Adopt Conformation of Mutant PrP<sup>res</sup>.** Almost all patients with inherited prion diseases are heterozygotes, harboring one pathogenic mutant and one WT *PRNP* allele, and therefore interactions between mutant and WT PrP molecules could affect the expression of disease phenotype. However, the nature of such interactions is not yet well understood. In some instances of familial prion disease, PrP amyloid plaques and fibrils have been found to be composed solely of mutant PrP,<sup>29–31</sup> and in one study of FFI patient samples mutant, but not WT, PrP<sup>res</sup> was detected following a series of detergent extractions and PK digestion.<sup>32</sup> Although to our knowledge misfolding of WT PrP has not yet been demonstrated in FFI patient samples, the existence of individuals who develop sporadic fatal insomnia (sFI) in the absence of a mutant allele, and the similarity of the PrP conformation found in sFI and FFI<sup>33</sup> suggest that WT PrP can adopt the pathogenic conformation responsible for fatal insomnia disease that is favored by the D177N mutation. Moreover, several studies of other inherited prion diseases, including fCJD caused by the E200 K mutation, demonstrate misfolding of both mutant and WT gene products,<sup>32,34,35</sup> and misfolding of WT PrP has been implicated in producing phenotypic heterogeneity among Gerstmann-Sträussler-Scheinker disease (GSS) patients carrying the P102L mutation.<sup>36</sup>

In our experiments, the spontaneously produced D177N recPrP<sup>res</sup> conformation could be easily distinguished from that of infectious recPrP<sup>Sc</sup> because the PK-resistant core displayed increased mobility as a doublet on SDS-PAGE. A similar conformation was always produced whether D177N recPrP substrate was subjected to sPMCA with or without infectious recPrP<sup>Sc</sup> seed or cofactors, indicating that the mutation is the sole determinant of D177N misfolding.

The largest PK-resistant fragment produced from D177N recPrP<sup>res</sup> in this *in vitro* study was a ~16 kDa, C-terminal peptide, and a fragment of similar *M<sub>r</sub>* was produced after PK-digestion of E199K recPrP<sup>res</sup>. These PK-resistant fragments are smaller than those derived from FFI and fCJD patient samples<sup>37,38</sup> and, importantly, do not exhibit the well described 2 kDa shift in *M<sub>r</sub>* that has been used to distinguish between the *in vivo* FFI- and fCJD-associated PrP conformations,<sup>37,38</sup> suggesting that PrP post-translational modifications and/or interactions with specific cellular factors must further direct the pathogenic misfolding pathway into the final disease-associated PrP conformations *in vivo*. Despite the fact that our *in vitro* conversion system does not fully recapitulate the PrP misfolding that must occur in patients with inherited prion disease, using a chemically defined system allowed us to study the molecular interactions between different PrP sequences and cofactor molecules that lead to spontaneous protein misfolding and cross-propagation in ways that would not be possible *in vivo*.

The unique Western blot pattern of the D177N recPrP<sup>res</sup> conformation allowed us to test whether it could cross-propagate into WT substrate. Interestingly, and as is diagrammed in Figure 7B, the results showed that the mutant recPrP<sup>res</sup> conformation could indeed be propagated into WT recPrP substrate, but only in the presence of a partially purified phospholipid cofactor preparation. The observation that WT recPrP substrate is able to adopt a recPrP<sup>res</sup> conformation initially created by a mutant seed is consistent with the results

of experiments in transgenic mice expressing mouse–human chimeric WT PrP inoculated with either FFI or fCJD prions.<sup>38</sup>

It is important to note that although D177N and E199K recPrP<sup>res</sup> displayed the ability to function as seeds for the conversion of WT recPrP substrate, we did not test whether these misfolded PrP conformers are bona fide prions, capable of causing disease with high specific infectivity upon injection into WT animals. Indeed, it is known that autocatalytic PrP conformers can display significant variability in infectious activity,<sup>6</sup> although the structural and functional basis of this variability remains unknown.

**Cross-Propagation of Mutant PrP Misfolding into WT Substrate Requires a Novel Phospholipid Cofactor.** The cofactor preparation that facilitated the cross-propagation of D177N recPrP<sup>res</sup> into WT recPrP substrate was originally purified from brain tissue by following activity that promoted infectious PrP<sup>Sc</sup> propagation *in vitro*.<sup>5</sup> Quantitative analysis of this preparation revealed that it contained only a mixture of five phospholipids, with PE and PC being the most abundant species. Of these, only PE displayed the ability to facilitate infectious recPrP<sup>Sc</sup> propagation. Moreover, sPMCA reactions with PE as the sole cofactor always produced a single, unique infectious recPrP<sup>Sc</sup> strain, regardless of input seed (in contrast to sPMCA reactions with crude brain homogenate substrate, which maintain the strain characteristics of the input seed). These results suggested that specific sets of cofactor molecules might be required to replicate specific PrP conformations.

Consistent with and extending this hypothesis, and shown schematically in Figure 7B, we observed in our current experiments that the ability of WT recPrP substrate to adopt and propagate the mutant D177N recPrP<sup>res</sup> conformation required components of the cofactor preparation other than PE. Pure PE failed to propagate the mutant conformation, while pure PC propagated a similar recPrP<sup>res</sup> conformation whose SDS-PAGE migration pattern differed subtly from that of D177N recPrP<sup>res</sup>. These results show for the first time that different cofactor molecules can facilitate the propagation of different conformations of WT PrP. It is likely that the accurate *in vitro* replication of the recPrP<sup>res</sup> conformer facilitated by the partially purified cofactor preparation depends on a particular combination of the component phospholipids, although we cannot exclude the possibility that trace, non-phospholipid components may also play a role.

Importantly, the findings described here for D177N recPrP were reproduced by analogy with a second pathogenic PrP mutation, E199K, but not with the M128V polymorphic substitution. The fact that two different PrP mutations associated with familial prion disease drive the production of similar, cofactor- and seed-independent, autocatalytic PrP conformers suggests that pathogenic mutations destabilize the  $\alpha$ -helical structure of PrP<sup>C</sup> in similar ways.

**Cofactor Molecules Antagonize WT PrP Inhibition of Mutant PrP Misfolding.** In the absence of cofactor molecules, we observed that WT recPrP in trans inhibits the spontaneous formation of D177N recPrP<sup>res</sup>, as represented in Figure 7C. That homotypic interactions can directly interfere with PrP misfolding has previously been demonstrated *in vitro*.<sup>39,40</sup> The heteroallelic inhibition observed in this study is effective when WT recPrP is present in a 3:1 molar excess of D177N recPrP. There is, in fact, evidence to suggest that this ratio may approach the natural stoichiometry of *PRNP* gene products found in FFI patient tissues: knock-in FFI mouse studies reported by Jackson et al. demonstrate that steady-state D177N



PrP expression is significantly reduced relative to endogenous WT PrP.<sup>9,10</sup> Therefore, in the heterozygote setting that is typical of FFI patients, it is likely that WT PrP molecules outnumber mutant PrP molecules by a substantial amount.

Interestingly, heteroallelic inhibition of PrP misfolding was antagonized by addition of cofactor, as is also diagrammed in Figure 7C. These results suggest that in individuals carrying the FFI mutation, the rate of PrP misfolding may be negatively influenced by interactions between the mutant and WT PRNP gene products, and positively influenced by interactions between PrP and cofactor molecules. A fragile balance between these opposing processes may provide an explanation for the highly variable time to disease onset (age 23–73 years) seen in FFI.<sup>41,42</sup>

Thus, cofactor molecules display two activities in vitro that might accelerate inherited prion disease in the typical heterozygote setting: (1) promote misfolding of WT PrP and (2) relieve WT PrP-mediated (heteroallelic) inhibition of mutant PrP misfolding.

In summary, our studies show that mutant PrP molecules are intrinsically unstable and can misfold spontaneously in the absence of other molecules. However, both WT PrP and cofactor molecules control the ability of mutant PrP to misfold and propagate its misfolded conformation in vitro. Taken together, these results suggest that the progression and clinical severity of inherited prion diseases might be influenced by the interplay of mutant, WT, and cofactor molecules within the membrane environment of neurons.

## ■ ASSOCIATED CONTENT

### ■ Supporting Information

Circular dichroism spectrum of D177N recPrP, and Western blot images of experiments describing the temperature, pH, and sonication dependence of spontaneous D177N recPrP<sup>res</sup> production; role of cofactor and seed in the formation and propagation of E199K recPrP<sup>res</sup> and M128V recPrP<sup>Sc</sup>; epitope mapping of D177N recPrP<sup>res</sup> PK-digestion products. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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## ■ ABBREVIATIONS

PrP, prion protein; WT, wild-type; PE, phosphatidylethanolamine; FFI, fatal familial insomnia; fCJD, familial Creutzfeldt-Jakob disease; MoPrP, mouse PrP; sPMCA, serial protein misfolding cyclic amplification; CD, circular dichroism; PLC,

phospholipase C; PK, proteinase K; MES, 2-(N-morpholino)-ethanesulfonic acid; *M<sub>r</sub>*, relative mobility; PC, phosphatidylcholine; mAb, monoclonal antibody; sFI, sporadic fatal insomnia; GSS, Gerstmann-Sträussler-Scheinker disease

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