

Developing a Method for *In Vitro* Conversion of Recombinant Mule Deer Prion Protein

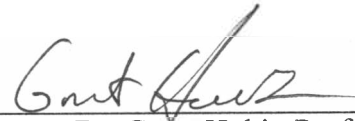
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Abstract

Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy (TSE) occurring in free-ranging and captive populations of deer and elk. CWD is thought to involve a misfolded protein called the prion protein. The misfolding mechanism remains unknown, but the conversion results from misfolded PrP^{SC} contacting normal PrP^C, and inducing a conformational change of PrP^C to PrP^{SC}. Previous studies have shown the normal cellular PrP^C, isolated from mule deer brain, has the ability to misfold *in vitro* when incubated with misfolded PrP^{SC}. Instead of using deer brain as a source of the normal cellular form of the prion protein, this project examined the use of recombinant mule deer prion protein expressed in High 5 insect cells. The cells were infected with a recombinant baculovirus that encodes and expresses the recombinant mule deer prion protein, rPrP^C. Developing *in vitro* conversion methods for the recombinant form of the prion protein will enable future studies that use this substrate to probe the mechanism of the misfolding process. Two methods, nondenaturing and protein-misfolding-cyclic-amplification (PMCA), have been devised to study the misfolding process *in vitro*. The observations reported here suggest that the recombinant mule deer prion protein was converted to the misfolded isoform by both nondenaturing and PMCA methodologies.

Introduction

Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy (TSE) occurring in free-ranging and captive populations of deer and elk (Gross and Miller, 2001). CWD is related to other diseases such as bovine spongiform encephalopathy (BSE), scrapie in sheep, and Creutzfeldt-Jakob Disease in humans (Prusiner, 1991). These diseases have a similar pathology and causative agent.

The infectious agent in these diseases is thought to be a misfolded protein called the prion protein (Prusiner, 1991). An important event in prion infection is the conformational conversion of PrP^C (normal cellular form) into PrP^{SC} (infectious cellular form) (Prusiner, 1991). Through pulse-chase experiments with scrapie-infected cultured cells, Prusiner (1991) observed that conversion is a posttranslational event, converting the protease-sensitive PrP^C to the infectious, protease resistant PrP^{SC} isoform. The detailed mechanism of this conversion remains unknown (Prusiner, 1991). However, the conversion results from the PrP^{SC} contacting PrP^C, and inducing a conformational change of PrP^C to PrP^{SC} (Prusiner, 1991).

Two different methods have been devised to study the *in vitro* conversion of PrP^C to PrP^{SC}. Lucassen et al. (2003) developed the nondenaturing process. This process calls for directly combining PrP^C brain homogenate with PrP^{SC} brain homogenate, and then the level of conversion of PrP^C to PrP^{SC} can be measured (Lucassen et al., 2003). Saborio et al. (2001) developed the more efficient protein-misfolding-cyclic-amplification (PMCA) process; this process allows for the rapid misfolding of PrP^C to PrP^{SC}, through cycles of incubation followed by sonication. Sonication allows for breakage of PrP^{SC} polymers,

forming new, smaller PrP^{SC} aggregate seeds, allowing an amplified misfolding process to continue (Saborio et al., 2001).

Instead of using deer brain as my source of PrP^C, I proposed to use recombinant mule deer prion protein expressed in High 5 insect cells, which are infected with a recombinant baculovirus that encodes and expresses the mule deer prion protein, rPrP^C. Using the recombinant mule deer prion protein allows for the direct study of the protein, therefore limiting other factors in the brain which may contribute to the misfolding process. Iniguez et al. (2000) used a baculovirus system to express the hamster prion protein. This recombinant hamster prion protein, PrP^C, was combined with PrP^{SC}, using a non-denaturing approach (Iniguez, 2000). This recombinant prion protein demonstrated the conversion of PrP^C to the misfolded, protease resistant form, PrP^{SC}, but the *in vitro* conversion process was only 25-30% efficient (Iniguez, 2000).

The purpose of the present study is two-fold. First, the ability to convert recombinant mule deer prion protein, rPrP^C to PrP^{SC} using the non-denaturing approach was investigated. Second, the recombinant mule deer prion protein conversion process was investigated using the PMCA approach. This would be the first successful demonstration of this process using recombinant, mule deer prion protein, rPrP^C. If the recombinant mule deer prion protein, rPrP^C, proves to be a suitable substrate in the conversion process and the conversion proves successful, then further studies by others can be performed to probe the mechanism of the conversion of PrP^C to PrP^{SC} in CWD.

I hypothesize that the recombinant mule deer prion protein, rPrP^C, will be converted into the misfolded PrP^{SC} when subjected to both the nondenaturing and PMCA *in vitro* conversion assays.

Materials and Methods

Maintenance of Cell Line

High 5 insect cells were maintained in 1X Express Five SFM Serum Free Medium supplemented with glutamine, penstrep antibiotics, and fungizone. Cells were passed when they were confluent. Confluency was typically obtained after 3 to 4 days of incubation at 27 °C. To pass cells, the UV light in the sterile hood had to be turned on for 4 hours and media had to be removed from the refrigerator ½ hour prior to use. The sterile hood was wiped down with 70% ethanol just prior to beginning. Hands and forearms were washed thoroughly with soap and water, and while passing cells, hands and forearms were periodically washed with 70% ethanol. This ensures a clean and sterile working environment. Cell flasks were wiped down with the 70% ethanol and placed into the sterile hood. The cells adhered to the surface of the flask during their growth period. Cells were washed away from the surface of the flask in order to pass them. This was done by tilting the flask upright, collecting the media in the bottom of the flask with an automatic pipette (sterile), and gently passing the collected media over the cells until they were completely removed from the surface of the flask. In the sterile hood, 4.5 mL of new media was placed into a new sterile flask and 0.5 mL of the washed cells was added, bringing the total volume to 5.0 mL. This was preformed every 3-4 days for the duration of the project.

Infecting Cells with Virus

The same sterile techniques as were used in maintaining the cell line were used to infect cells with the virus. The virus used was a recombinant baculovirus encoding the

mule deer prion protein. Before infection, the cells were observed under an inverted microscope to check for confluency and overall health of the cells. Only the flasks of healthy, confluent cells were used for infection. In the sterile hood (a different hood from that used for cell passage), the virus was prepared by pipetting 1.0 mL of media for each flask to be infected into a 15 mL sterile centrifuge tube. Twenty μ L of virus was added per each 1.0 mL of media in the centrifuge tube and the virus/media tube was gently mixed. Next, the media of the cells to be infected was removed and the 1.0 mL virus/media solution was gently added to the flask. The flasks were placed on a rocking platform for one hour at room temperature so that the media could slowly spread over the cells, ensuring their infection. After rocking, the cells were returned to the sterile hood and 2.0 mL of media were added to each flask, bringing the total volume to 3.0 mL per flask. The newly infected cells were placed into the incubator, at 27 °C. Optimal protein expression occurred 42-48 hours post infection.

Preparing Intact Insect Cell Samples for Immunoblot Analysis

Infected cells were dislodged from the flask and placed into a sterile 15 mL centrifuge tube. Cells were spun for 5 minutes at 1380 x G and the supernatant was discarded. Cells were gently resuspended in 2.0 mL of phosphate buffered saline (PBS) and spun again for 5 minutes at 1380 x G and the supernatant was discarded. Cells were resuspended in 2.0 mL of PBS again and spun for a third time for 5 minutes at 1380 x G and the supernatant was discarded. Cells were resuspended in 0.5 mL of PBS. Samples were treated with SDS sample buffer, boiled for 10 minutes, and loaded on a gel for immunoblotting analysis.

Electrophoresis and Immunoblotting

Pre-poured NuPage 12% Bis-Tris gels (Invitrogen) were used for electrophoresis. After loading the samples, gels were run at 200 V for approximately 1 hour using Invitrogen equipment. Blotting membranes (PVDF) were cut and soaked in methanol for 15 seconds and then soaked in deionized water for 2 additional minutes. The blotting apparatus was assembled and transfer of proteins from the gel to the PVDF membrane occurred at 30 V for 1-1.5 hours. Membranes were then incubated in 30 mL of blocking buffer (5% w/v nonfat dry milk in TBST) ((1 L TBST) (2.4 g Tris, 8.2 g NaCl, PH to 7.5, 1 ml Tween20, fill to 1 L with deionized water)) for 1 hour. Blocking buffer was removed and membranes were briefly washed with TBST. Membranes were incubated with the primary antibody (20 μ L of anti-Prion monoclonal antibody, SAF-32 in 4.0 mL of dilution buffer (1% w/v nonfat dry milk in TBST)) and heat sealed and incubated at 4°C overnight. In preparation for the secondary antibody, the membrane was removed from the heat sealed pouch and washed for 10 minutes in 20 mL of TBST for three consecutive washes. The secondary antibody (2 μ L of anti-mouse IgG in 15 mL of blocking buffer) was added to the membrane at room temperature on the rocking platform for 1 hour. Secondary antibody was removed and the membrane was washed 3 more times in 20 mL of TBST for 10 minutes per wash. The membranes were then washed twice in 25 mL of assay buffer (5 mL CDP-Star Assay Buffer in 45 mL of deionized water) for 2 minutes. Next, 2-3 mL of CDP-Star Substrate was added directly onto the membrane and allowed to sit for 5 minutes. Chemiluminescent imaging was done using Molecular Imaging software and a Kodak 1500 Molecular Imager.

Preparation of Insect Cell Membranes

Preparation of insect cell membranes for nondenaturing and PMCA conversions was adopted from Kurt et al. (2007). The following modifications were made because insect cells were used instead of deer brain. Sample preparation was the same as described for the preparation for immunoblot analysis (above), except the following steps were omitted. Cells were not resuspended in 0.5 mL of PBS, treated with SDS sample buffer, or boiled for 10 minutes.

To prepare membranes for nondenaturing conversions, nondenaturing buffer (1.0 mL of PBS plus complete protease inhibitor (CPI)) were added per flask of cells. For PMCA conversions, PMCA buffer (1.0 mL of PBS, 1% Triton X-100, 5 mM EDTA, 150 mM NaCl, and CPI) were added per flask of cells.

A Tenbroek homogenizer was then used to rupture the cells of each homogenate. Twelve passes were done with the homogenizer for each homogenate and then the cells were viewed under an inverted microscope to ensure they were completely ruptured. Nondenaturing homogenates were centrifuged for 30 seconds at 269 G. PMCA homogenates were centrifuged for 90 seconds at 1470 G. The membrane-containing supernatants were collected in 250 μ L aliquots, labeled, and stored at -50 °C.

Nondenaturing and PMCA conversions

The first attempts at the nondenaturing conversion assay were adopted from Kurt et al. (2007). Some modifications were made. A series of three samples were produced and used as experimental, positive, and negative controls (Table 1).

Table 1. Variables tested for first attempts at the nondenaturing *in vitro* conversion assay.

Tube #1 Positive Control	50 μ L of a 1:200 dilution of 10% CWD PrP ^{SC} deer brain homogenate diluted in PBS was added to 50 μ L of a 1:200 dilution of 10% non-infected deer PrP ^C brain homogenate diluted in PBS.
Tube #2 Negative Control	50 μ L of a 1:200 dilution of 10 % non-infected deer PrP ^C homogenate was added to 50 μ L of 1:200 dilution of rPrP ^C insect cell homogenate; both dilutions were done in PBS.
Tube #3 Experimental	50 μ L of a 1:200 dilution of 10% CWD PrP ^{SC} was added to 50 μ L of 1:200 dilution of rPrP ^C insect cell homogenate; both dilutions done in PBS.

The second attempts at the nondenaturing conversion assay were modified from Kurt et al. (2007). A series of six samples were produced and used as experimental, positive, and negative controls (Table 2).

Table 2. Variables tested for second attempts at the nondenaturing *in vitro* conversion assay.

Tube #1 Positive Control	10 μ L of 10% CWD PrP ^{SC} deer brain homogenate was added to 90 μ L of 10% non-infected deer PrP ^C brain homogenate. Final dilution was 1:10.
Tube #2 Negative Control	10 μ L of 10% non-infected deer PrP ^C homogenate was added to 90 μ L of 10% rPrP ^C insect cell homogenate. Final dilution was 1:10.
Tube #3 Experimental	10 μ L of 10% CWD PrP ^{SC} deer brain homogenate was added to 90 μ L of 10% rPrP ^C insect cell homogenate. Final dilution was 1:10.
Tube #4 Positive Control	5 μ L of 10% CWD PrP ^{SC} deer brain homogenate was added to 95 μ L of 10% non-infected deer PrP ^C brain homogenate. Final dilution was 1:20.
Tube #5 Negative Control	5 μ L of 10% non-infected deer PrP ^C homogenate was added to 95 μ L of 10% rPrP ^C insect cell homogenate. Final dilution was 1:20.
Tube #6 Experimental	5 μ L of 10% CWD PrP ^{SC} deer brain homogenate was added to 95 μ L of 10% rPrP ^C insect cell homogenate. Final dilution was 1:20.

Samples were incubated in a Tomy mixer at 37 °C for 8 hours. Samples were then treated with 50 μ g/mL of proteinase K (PK) and shaken for an hour. After the hour of PK digestion, PK digest was stopped by adding reducing agent and sample buffer to the samples. Samples were subjected to immunoblot analysis. PrP^{SC} is partially resistant to proteolysis by PK. This property is used as a means of monitoring the conversion process.

The first attempts at the PMCA conversion assay were adopted from Kurt et al. (2007). Some modifications were made. A series of eight samples were produced and used as experimental, positive, and negative controls (Table 3).

Table 3. Variables tested for first attempts at the PMCA *in vitro* conversion assay.

Tube #1 Positive Control	10% CWD PrP ^{SC} deer brain homogenate was diluted to a final dilution of 1:9000 in 10% non-infected PrP ^C brain homogenate. Final volume was 60 μ L. NOT sonicated.
Tube #2 Positive Control	10% CWD PrP ^{SC} deer brain homogenate was diluted to a final dilution of 1:9000 in 10% non-infected PrP ^C brain homogenate. Final volume was 60 μ L. Sonicated.
Tube #3 Positive Control	10% CWD PrP ^{SC} deer brain homogenate was diluted to a final dilution of 1:27,000 in 10% non-infected PrP ^C brain homogenate. Final volume was 60 μ L. Sonicated.
Tube #4 Experimental	10% CWD PrP ^{SC} deer brain homogenate was diluted to a final dilution of 1:9000 in rPrP ^C insect cell homogenate. Final volume was 60 μ L. NOT sonicated.
Tube #5 Experimental	10% CWD PrP ^{SC} deer brain homogenate was diluted to a final dilution of 1:9000 in rPrP ^C insect cell homogenate. Final volume was 60 μ L. Sonicated.
Tube #6 Experimental	10% CWD PrP ^{SC} deer brain homogenate was diluted to a final dilution of 1:27,000 in rPrP ^C insect cell homogenate. Final volume was 60 μ L. NOT sonicated.
Tube #7 Experimental	10% CWD PrP ^{SC} deer brain homogenate was diluted to a final dilution of 1:27,000 in rPrP ^C insect cell homogenate. Final volume was 60 μ L. Sonicated.
Tube #8 Negative Control	10 % non-infected PrP ^C brain homogenate was diluted to final dilution 1:1000 in rPrP ^C insect cell homogenate. Final volume was 60 μ L. Sonicated.

The second PMCA conversion assay was modified from Kurt et al. (2007). A series of eight samples were produced and used as experimental, positive, and negative controls (Table 4).

Table 4. Variables tested for second attempts at the PMCA *in vitro* conversion assay.

Tube #1 Positive Control	6 μ L of 10% CWD PrP ^{SC} deer brain homogenate was added to 54 μ L 10 % non-infected PrP ^C brain homogenate. Final dilution was 1:10. NOT sonicated.
Tube #2 Positive Control	6 μ L of 10% CWD PrP ^{SC} deer brain homogenate was added to 54 μ L 10 % non-infected PrP ^C brain homogenate. Final dilution was 1:10. Sonicated.
Tube #3 Positive Control	3 μ L of 10% CWD PrP ^{SC} deer brain homogenate was added to 57 μ L 10 % non-infected PrP ^C brain homogenate. Final dilution was 1:20. Sonicated.
Tube #4 Negative Control	60 μ L 10 % non-infected PrP ^C brain homogenate. Sonicated.
Tube #5 Experimental	6 μ L of 10% CWD PrP ^{SC} deer brain homogenate was added to 54 μ L rPrP ^C insect cell homogenate. Final dilution was 1:10. NOT sonicated.
Tube #6 Experimental	6 μ L of 10% CWD PrP ^{SC} deer brain homogenate was added to 54 μ L rPrP ^C insect cell homogenate. Final dilution was 1:10. Sonicated.
Tube #7 Experimental	3 μ L of 10% CWD PrP ^{SC} deer brain homogenate was added to 57 μ L rPrP ^C insect cell homogenate. Final dilution was 1:20. Sonicated.
Tube #8 Negative Control	6 μ L 10 % non-infected PrP ^C brain homogenate was added to 54 μ L rPrP ^C insect cell homogenate. Final dilution was 1:10. Sonicated.

The samples were subjected to PMCA and treated with PK following the conditions described by Kurt et al. (2007). PK digestion was stopped by adding sample buffer and the reducing agent. Samples were analyzed by immunoblotting analysis.

Figure 1 provides a summary of the experimental design for this study.

Results

Table 5 summarizes the samples tested throughout the duration of this study. Table 6 summarizes the different parameters tested throughout the study. The numbers in table 6 represent ratios of PrP^{CWD} to recombinant rPrP^C and PrP^{CWD} to deer brain PrP^C. Table 7 provides a summary of positive and negative results. The protocol followed by Kurt et al. (2007) provided negative results, so modifications were made. These modifications then produced positive results (Table 7).

Figure 2 shows prion protein expression levels of intact High 5 insect cells after the recombinant baculovirus that encodes and expresses the mule deer prion protein, PrP^C, had been added. The expression levels of the recombinant mule deer prion protein, rPrP^C, were then compared to prion protein in mule deer brain to ensure that expression levels were comparable. Expression levels were comparable (data not shown).

Next, insect cell membranes containing the rPrP^C were prepared for nondenaturing and PMCA. Figure 3 shows the recovery of rPrP^C from insect cells and PrP^C from mule deer brain for the nondenaturing membranes. Both recombinant mule deer prion protein expressed in insect cells and mule deer brain produced similar expression levels of prion protein, using 10 µL of each sample, respectively. Likewise, Figure 4 shows that PMCA insect and deer brain membranes yielded similar levels of prion protein, indicated by intensities of the banding. Ten µL of each sample of PMCA membrane were loaded, respectively.

Figure 5 shows the results from the first attempts for the nondenaturing *in vitro* conversion assay. A 1:200 ratio of PrP^{CWD} to PrP^C was used for both insect cells and deer brain. The absence of banding indicates that the first attempts at the nondenaturing

assay were unsuccessful and provided negative results. Likewise, figure 6 shows that the first attempts for the PMCA *in vitro* conversion assay also proved to be unsuccessful due to the lack of banding, providing negative results. A series of different dilutions, 1:27,000; 1:9000; and 1:1,000, was used for the experimental.

After modifying the methods from Kurt et al. (2007), positive results were achieved for both the nondenaturing and PMCA *in vitro* conversion assays. Lane 2 of figure 7 suggests that a 1:20 ratio of PrP^{CWD} to PrP^C allowed the PrP^C from mule deer brain to misfold in the nondenaturing *in vitro* conversion assay. Faint banding in lane 3 of figure 7 also suggests recombinant mule deer prion protein had the ability to misfold when a 1:20 ratio of PrP^{CWD} to rPrP^C was added, and treated with PK. Forty μ L of each sample was loaded, respectively.

Figure 8 suggests the PMCA *in vitro* conversion assay provides positive results. Lanes 2 and 3 of figure 8 compare a 1:10 ratio of PrP^{CWD} to deer brain PrP^C. Lane 2 was not subjected to PMCA and lane 3 was subjected PMCA. A darker band in lane 3, as compared to lane 2, suggests the PMCA *in vitro* conversion assay provided a larger quantity of PrP^{CWD} conversion. Forty μ L of sample were loaded for lanes 2 and 3, respectively. Likewise, lanes 4, 5, and 6, provided positive results. Lanes 4 and 5 contained 40 μ L of a 1:10 ratio of PrP^{CWD} to rPrP^C. Lane 4 was not subjected to PMCA and lane 5 was subjected to the PMCA *in vitro* conversion assay. Lane 6 contained a 1:20 ratio of PrP^{CWD} to rPrP^C. The presence of banding in lanes 4, 5, and 6 suggests that the recombinant mule deer prion protein expressed in High 5 insect cells has the ability to misfold to the protease resistant PrP^{CWD} form. Forty μ L of each sample were loaded for each sample in lanes 4, 5, and 6.

Table 5. Samples tested during the *in vitro* conversion assays.

Variables	
positive control	$\text{PrP}^{\text{CWD}} + \text{dbPrP}^{\text{C}}$
negative control	$\text{dbPrP}^{\text{C}} + \text{rPrP}^{\text{C}}$
experimental	$\text{PrP}^{\text{CWD}} + \text{rPrP}^{\text{C}}$

Note: PrP^{CWD} stands for the misfolded, protease-resistant, infectious mule deer prion protein. dbPrP^{C} stands for normal cellular form of the mule deer prion protein expressed in deer brain homogenate. rPrP^{C} stands for the recombinant normal cellular form of the mule deer prion protein expressed in insect cells.

Table 6. Parameters tested during *in vitro* conversion assays.

Variable Tested					
Ratio of PrP ^{CWD} to rPrP ^C (Nondenaturing)	1:200	1:20	1:10		
Ratio of PrP ^{CWD} to rPrP ^C (PMCA)	1:27,000	1:9,000	1:1,000	1:20	1:10

Table 7. Positive and negative results for each assay tested.

Nondenaturing	1:200	1:20	1:10
Positive Results		+	+
Negative Results	+		

PMCA	1:27,000	1:9,000	1:1,000	1:20	1:10
Positive Results				+	+
Negative Results	+	+	+		

Experimental Design

Establish and maintain High 5 insect cell line



Infect High 5 insect cells with a recombinant baculovirus that encodes and expresses the mule deer prion protein, PrP^C



Check for prion production by immunoblotting analysis



Preparation of nondenaturing and PMCA insect cell membranes: check recovery of prion protein by immunoblotting



Expose both PrP^C insect cell homogenates to PrP^{Sc} deer brain homogenates



Treat samples with proteinase K, reducing agent, and sample buffer



Subject samples to immunoblotting analysis



Successful conversion determined based on the appearance of banding (misfolded PrP^{Sc} is proteinase K resistant)

Figure 1. An outline of the experimental design.

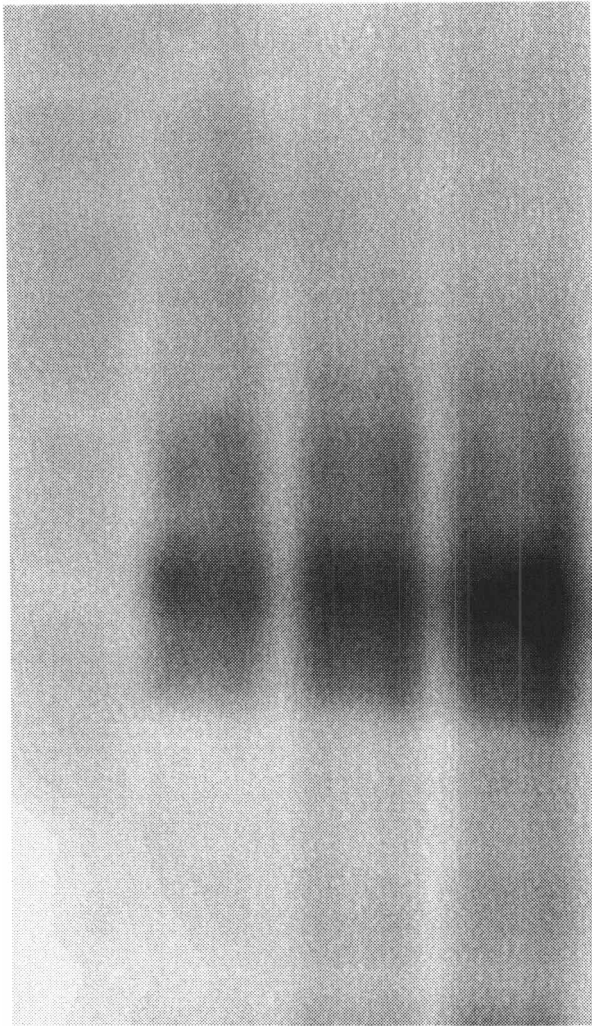


Figure 2. Expression levels of recombinant rPrP^C in intact insect cells. From left to right, molecular weight marker, 3 μ L, 10 μ L, and 15 μ L of intact High 5 insect cells, respectively.

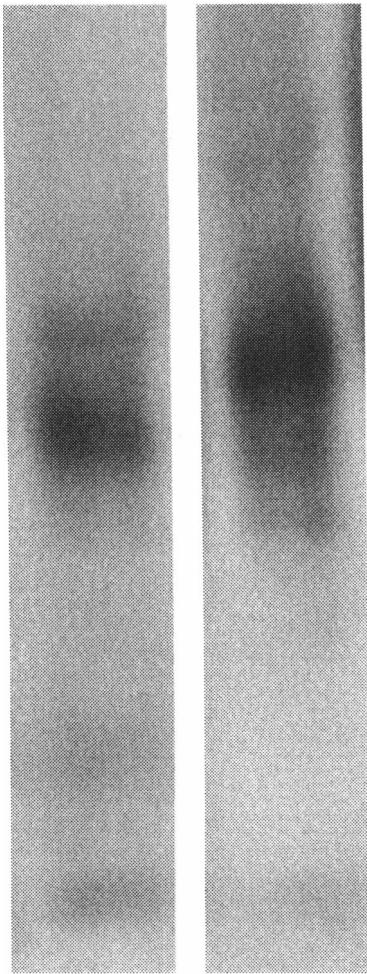


Figure 3. Recovery of PrP^C from nondenaturing membranes. From left to right, 10 μL of High 5 insect cell nondenaturing membrane prep, and 10 μL of deer brain nondenaturing membrane prep.

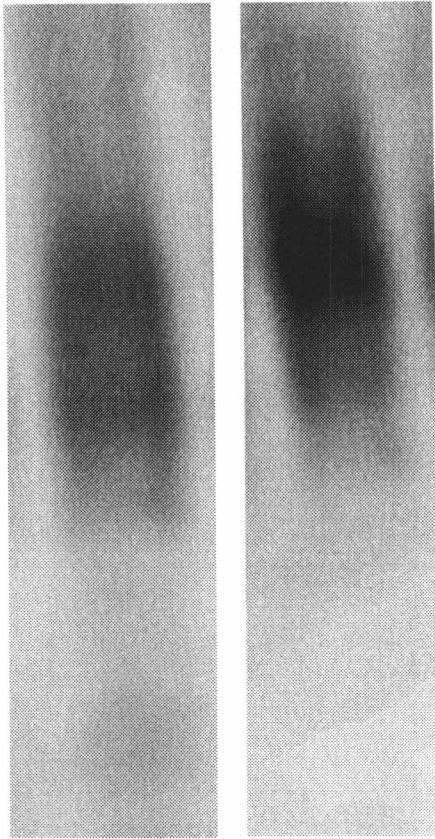
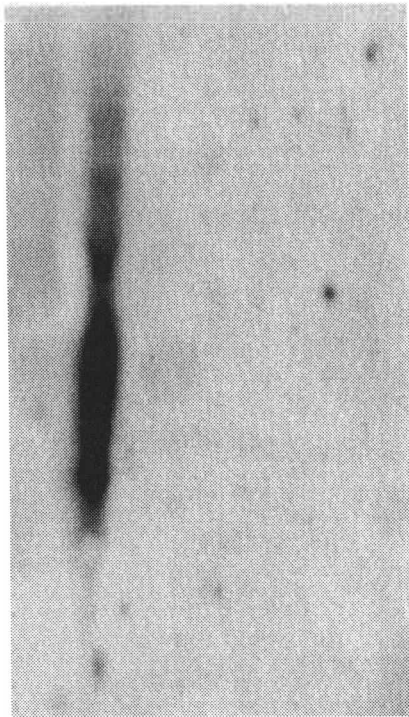


Figure 4. Recovery of PrP^C from PMCA membranes. From left to right, 10 μ L of High 5 insect cell PMCA membrane prep, and 10 μ L of PMCA deer brain membrane prep.



1 2 3 4 5

Figure 5. Negative results. 1:200 dilution of nondenaturing membranes.

Lane 1: molecular weight maker

Lane 2: 5 μ L of 10% deer brain homogenate

Lane 3 (Positive Control): 24 μ L of 1:200 ratio of PrP^{CWD} + dbPrP^C

Lane 4 (Negative Control): 24 μ L of 1:200 ratio of dbPrP^C + rPrP^C

Lane 5 (Experimental): 24 μ L of 1:200 ratio of PrP^{CWD} + rPrP^C

Samples from lanes 3-5 were treated with proteinase K. Any bands present would indicate misfolding to the protease resistant PrP^{Sc} form.

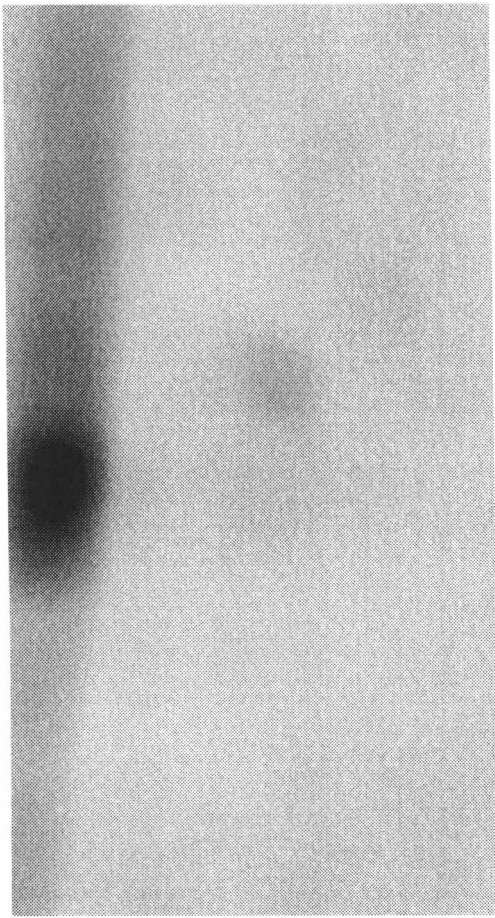


Figure 6. Negative results. 1:27,000, 1:9000, 1:1000 dilutions of PMCA membranes.

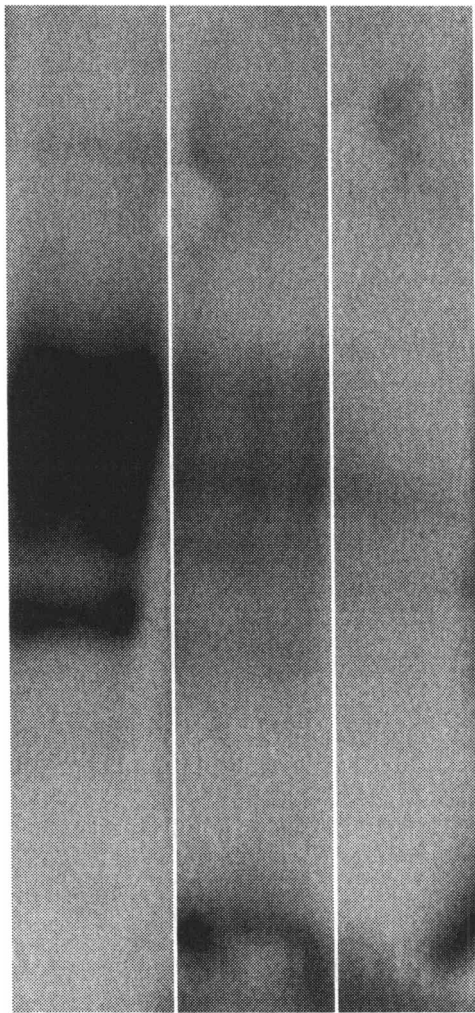
Lane 1: 2.5 μ L of 10% deer brain homogenate

Lane 2 (Experimental): 40 μ L of 1:1000 ratio of PrP^{CWD} + rPrP^C

Lane 3 (Experimental): 40 μ L of 1:9000 of ratio of PrP^{CWD} + rPrP^C

Lane 4 (Experimental): 40 μ L of 1:27,000 ratio of PrP^{CWD} + rPrP^C

Samples from lanes 2-4 were treated with proteinase K. Any bands present would indicate misfolding to the protease resistant PrP^{SC} form.



1 2 3

Figure 7. Positive results. 1:20 dilutions for nondenaturing membranes.

Lane 1: 1 μ L of 10% deer brain homogenate

Lane 2 (Positive Control): 40 μ L of 1:20 ratio of PrP^{CWD} + dbPrP^C

Lane 3 (Experimental): 40 μ L of 1:20 ratio of PrP^{CWD} + rPrP^C

Lanes 2-3 were treated with proteinase K. The visible bands indicate the presence of the misfolded, proteinase K resistant PrP^{SC}.

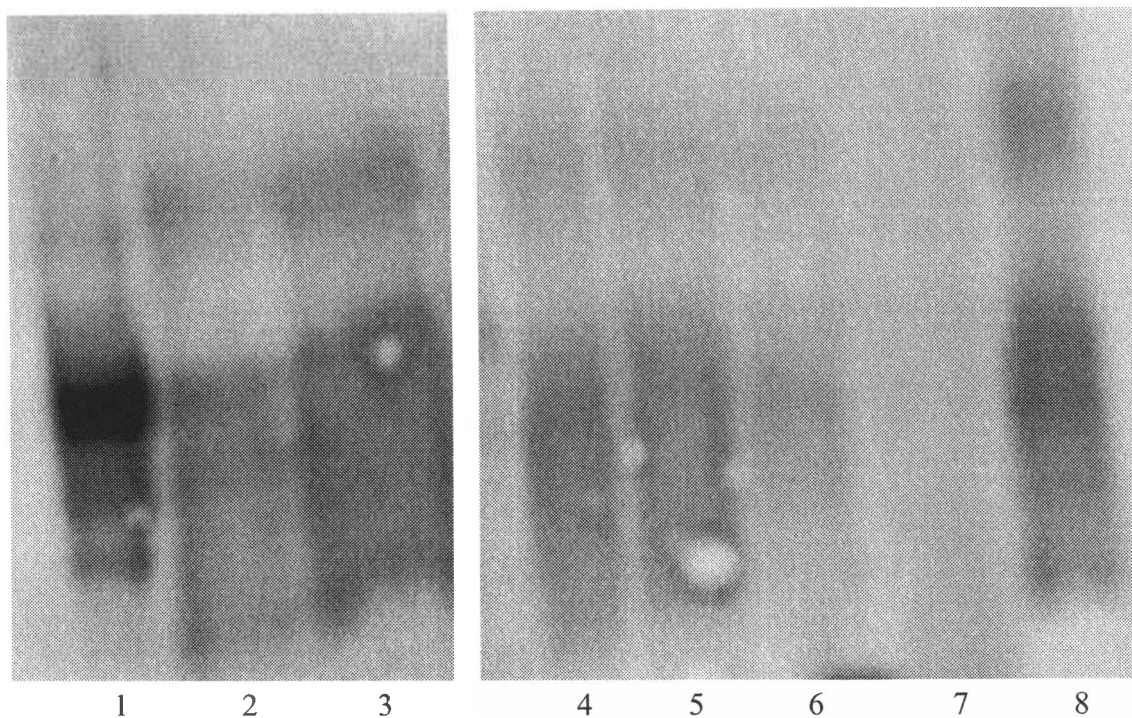


Figure 8. Positive results. 1:20 and 1:10 dilutions for PMCA membranes.

Lane 1: 1 μ L of 10% deer brain homogenate

Lane 2 (Positive Control, not subjected to sonication): 40 μ L of 1:10 ratio of PrP^{CWD} + dbPrP^C

Lane 3 (Positive Control, subjected to sonication): 40 μ L of 1:10 ratio of PrP^{CWD} + dbPrP^C

Lane 4 (Experimental, not subjected to sonication): 40 μ L of 1:10 ratio of PrP^{CWD} + rPrP^C

Lane 5 (Experimental, subjected to sonication): 40 μ L of 1:10 ratio of PrP^{CWD} + rPrP^C

Lane 6 (Experimental, subjected to sonication): 40 μ L of 1:20 ratio of PrP^{CWD} + rPrP^C

Lane 7 (Negative Control): 40 μ L of 1:10 ratio of dbPrP^C + rPrP^C

Lane 8: 10 μ L of PrP^{CWD}

Lanes 2-8 were treated with proteinase K. Immunoreactive bands indicate the presence of misfolded PrP^{SC}.

Discussion

These results suggest that recombinant mule deer prion protein expressed in High 5 insect cells has the ability to misfold to the protease resistance PrP^{SC} form. The first attempts at the nondenaturing and PMCA *in vitro* conversion assays produced negative results. Modifications were made by altering the ratios of PrP^C to PrP^{SC}, and the ratios of rPrP^C to PrP^{SC}. These modifications then produced positive results. However, more attempts with the new modifications will need to be run in order to produce quantifiable data. Once quantifiable data are obtained, future experiments may be performed to give further insight into the mechanism of prion protein conversion. In recent years, competing hypotheses have arisen as to what specific cellular factors contribute in the ability of prion proteins to misfold from the normal cellular form, PrP^C, to the misfolded, infectious cellular form, PrP^{SC}.

Taraboulos et al. (1995) introduced varying concentrations of lovastatin, a sterol synthesis inhibitor, to the ScN2_a cell line and concluded that increasing lovastatin is directly proportional in reducing the *in vitro* conversion of PrP^C to PrP^{SC}. This suggests that membrane cholesterol concentration can influence prion conversion. However, lovastatin can affect other cellular metabolites such as isoprenoids and can thus alter important cellular processes. This creates doubt about the interpretation of these results.

Insect cells do not synthesize cholesterol. By using an insect cell line to express the recombinant mule deer prion protein, rPrP^C, the cholesterol is provided in the media, unlike mammalian cells lines which can synthesize cholesterol. This will allow us to reduce cholesterol by reducing the cholesterol concentration in the growth medium. This approach will allow us to test the influence of membrane cholesterol without using sterol synthesis inhibitors.

Future experiments will involve incrementally depleting cholesterol concentrations in the cellular media, preparing nondenaturing and PMCA membrane samples, running the nondenaturing and PMCA *in vitro* conversion assays as describe above, and producing quantifiable data. The results from the two *in vitro* conversion assays can then be used to answer the question: how does depleting cholesterol concentrations in the cell affect the ability of the recombinant mule deer prion protein, rPrP^C, to misfold? These data may provide further insight into the misfolding mechanism of rPrP^C to PrP^{SC}.

My results support my original hypothesis which suggests the recombinant mule deer prion protein, rPrP^C, can be converted into the misfolded PrP^{SC} when subjected to both the nondenaturing and PMCA *in vitro* conversion assays.

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