Cell-Free Formation of Protease-Resistant Prion Protein

by

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ABSTRACT

The transmissible spongiform encephalopathies (TSEs) are a class of neurodegenerative diseases that are characterized by proteinaceous deposits in the brain. The deposits consist largely of an abnormal form of prion protein which is highly aggregated and resistant to degradation by proteases. The function of prion protein (PrP) is unknown and its normal form (PrP^C) is sensitive to protease digestion. Some of the TSEs include scrapie in sheep, mice, and hamsters, bovine spongiform encephalopahty, and Creutzfeldt-Jakob disease in humans. Animals with scrapie accumulate a disease-specific form of PrP designated PrP^{Sc}. The identity of the infectious agent of the TSEs is unclear. No conventional agent or disease-specific nucleic acid has been found and treatments that destroy most normal viruses have no effect. Based on that information the infectious particle has been surmized to consist solely of protein. The "protein only" theory has been strengthened by the discovery of PrP and that preparations of PrP^{Sc} are greatly enriched for infectivity. Previously, the simplest system producing protease-resistant PrP was cell culture. Here is described a system that produces protease-resistant PrP *in vitro.* This system was used to study the mechanism of the conversion of PrP^C to the protease-resistant form. A threshold concentration of aggregates of PrP^{Sc} was required for conversion. Guanidine-HCl solubilized PrP^{Sc} had no converting activity. The need for aggregates and a threshold concentration favors a seeded polymerization mechanism of conversion. The system was also used to study species barriers and different strains of scrapie. Different combinations of mouse and hamster PrPs were used in conversion reactions. The *in vitro* conversion results correlated with infectivity data on transmission. Scrapie strains with different PrP^{Sc} N-terminal protease cleavage sites were used with this system. These different cleavage sites were passed to newly-resistant PrP^C depending on which strain was in the reaction. This system provided evidence that a "protein only" theory is compatible with the properties of TSEs.

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List of abbreviations

BSE	bovine spongiform encephalopathy
CJD	Creutzfeldt-Jakob disease
CPC	cetyl pyridinium chloride
cpm	counts per minute
CTAB	cetyl trimethyl ammoinum bromide
DY PrP^{Sc}	drowsy strain of scrapie associated PrP
FFI	fatal familial insomnia
FTIR	Fourier-transform infrared spectroscopy
GAG	glycosaminoglycan
GdnHCl	guanidine hydrochloride
GdnSCN	guanidine thiocyanate
GPI	gylcosyl phohphotidylinositol
GSS	Gerstmann-Straussler-Schenker disease
HaPrP	hamster prion protein
HFIP	hexafluoroisopropanol
HPLC	high pressure liquid chromatography
HY PrP^{Sc}	hyper strain of scrapie associated PrP
MoPrP	mouse prion protein
MNB	mouse neuroblastoma
NMWL	nominal molecular weight limit
PIPLC	phosphatidylinositol-specific phospholipase C
PK	proteinase K
PNGase F	peptide N glycosidase F
PRNP	gene encoding PrP
PrP	prion protein
PrP^{Sc}	scrapie-associated prion protein
PrP^{C}	cellular prion protein
RML	Rocky Mountain Laboratories
SAF	scrapie associated fibrils
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Tg	transgenic
TME	transmissible mink encephalopathy
TSE	transmissible spongiform encephalopathy

Chapter 1

The Transmissible Spongiform Encephalopathies

The transmissible spongiform encephalopathies (TSEs) are a class of neurodegenerative diseases characterized by abnormal proteinaceous deposits in the brain. A wide range of mammals (including humans) are susceptible to various types of TSEs. Scrapie, the most widely studied TSE, is naturally present in sheep. Other TSEs in animals include bovine spongiform encephalopathy (BSE), transmissible mink encephalopathy (TME), and chronic wasting disease in elk and mule deer. TSEs in humans include Creutzfeldt-Jakob disease (CJD), Gerstmann-Straussler-Schenker syndrome (GSS), kuru, and fatal familial insomnia (FFI).

The TSEs are sporadic, infectious, and genetic diseases which makes them unique. CJD affects about one person per million per year with about 5% of the cases being caused by germ-line mutations (familial cases). CJD can be accidentally transmitted between people through medical procedures (iatrogenic transmission). Accidental transmissions have been caused by cornea transplants from CJD patients, contaminated surgical instruments, and contaminated growth hormones, for example. The unusual resistance of the infectious agent to normal sterilization procedures increases the likelihood of these instances, which account for about 1% of all CJD cases. More than 90% of CJD cases are sporadic and can not be traced to a mutation or specific source of infection.

CJD is characterized by dementia, ataxia (a loss of muscle coordination), and the formation of small holes in the brain known as spongiform degeneration.

This spongiform degeneration is the result of a loss of neurons. CJD is often accompanied by abnormal proteinaceous deposits known as amyloid plaques. The onset of CJD can be months to years after infection, but once the disease presents itself a gradual deterioration of mental capabilities ensues. The deterioration ends in death usually within a year of onset. Heparin sulfates have been shown to offer some protection to mice inoculated with scrapie,[1] but as of now there is no way to stop the deterioration and the disease is always fatal.

GSS is a familial version of CJD with different pathology. All GSS cases are accompanied by the formation of amyloid plaques[2] and it is transmissible. FFI, caused by a point mutation and polymorphism, results in incurable insomnia, selective atrophy of thalamic nuclei, and dysautonomia (abnormal functioning of the autonomic nervous system). FFI is much less characterized by spongiform degeneration than CJD,[3] but nevertheless has recently been shown to be transmissible.[4] Kuru was found to affect a small population in New Guinea that practiced ritual cannibalism. In 1976, D. Carlton Gadjusek won the Nobel Prize in medicine for the transmission of kuru to primates and the assumption that its spread occurred through the cannibalism. As the practice of cannibalism has ceased, the disease has been mostly eliminated.[5]

The most studied TSEs are scrapie, originally from sheep and goats, and BSE or "mad cow disease." Scrapie has been recognized for hundreds of years, but BSE became a problem in Great Britain in 1985. Scrapie is passed between sheep but the source of BSE is uncertain. One theory of the cause of the epidemic is that scrapie-infected sheep were processed and added to cattle feed. As BSE may have originated through cows exposed to sheep scrapie, the risk to humans eating infected beef is unknown. In Great Britain, it is now feared that cases of a new CJD strain may have been caused by ingestion of BSE. This report has

resulted in many countries banning British beef. More BSE information is presented in the introduction to chapter 4.

Early studies of the scrapie agent

As might be expected, the early studies assumed that the TSEs were transmitted through some conventional agent. Sigurdarson defined the TSEs as slow infections based upon studies of chronic diseases afflicting Icelandic sheep.[6] Slow infections have characteristics of acute and chronic diseases. In acute diseases, the host will either fight off the infection or die rapidly. Chronic diseases are associated with extended remission periods and then reoccurrence. The term slow infection does describe the TSEs regardless of the identity of the infectious agent and how the host fights an infection. The TSEs have a variable period of up to decades with no symptoms after infection, but the course of the disease after onset is rapid.

Hadlow later found pathological similarities between scrapie and kuru and suggested that the diseases may be related even though they were from different species.[7] In 1965, Gadjusek, Gibbs, and Alpers transmitted kuru to chimpanzees[8] and later transmitted CJD to chimpanzees and monkeys[9, 10] which showed that the diseases were related. Scrapie in sheep was previously known to be transmissible and this suggested that all TSEs were related. Chandler was able to transmit sheep scrapie to mice which greatly facilitated its study.[11] The use of mice to study scrapie was an improvement over sheep, but the course of the disease was still about a year. The development of a line of scrapie in Syrian golden hamsters[12, 13] aided studies by further reducing the time of the course of the disease. The bioassay for infectivity was further refined through the use of incubation time, the time from infection to the appearance of clinical symptoms, instead of death as an endpoint.[14]

Because the infectious agent of the TSEs had not been identified, studies were done that allowed speculation on its identity. The infectious agent was resistant to treatment by heat and acetylethyleneimine,[15] which would be expected to inactivate nucleic acid. Treating the infectious agent with small doses of ionizing radiation at 254 nm, which would have inactivated typical viruses, also did not affect the infectivity.[16] The large dose of 254 nm radiation required for inactivation suggested that the agent was smaller than most viruses. The involvement of nucleic acids in the infection process remains controversial today as does the identity of the agent. These treatments aimed at altering nucleic acids would not have affected the protein that was later purified from infectious preparations and postulated to be the infectious agent.

Purification of prion protein from infectious preparations

The "recent" studies of scrapie began in 1981 with the identification of scrapie associated fibrils by Merz and coworkers using electron microscopy (EM).[17] Normal mice did not contain the fibrils and later humans with CJD were also found to have them.[18] Originally these fibrils were classified as non-amyloid, but this was later shown to be incorrect.[19] Prusiner and coworkers purified a protein from scrapie brain extracts through low-speed centrifugation, precipitation from ethylene glycol and ammonium sulfate, enzymatic digestion, and sedimentation through a sucrose gradient.[20] The purified protein was named prion protein or PrP. Prion stands for "proteinaceous infectious particle." The purification procedure for PrP was shown to concentrate infectivity 100 to 1000 fold by subsequent bioassay using hamsters. The major constituent of the enzymatically digested fractions enriched for infectivity was a 27-30 kDa protein named PrP 27-30. PrP 27-30 also formed fibrils which were probably identical to those identified by Merz. At this time it was suggested that the infectious agent

in scrapie was made up solely of PrP. This "protein-only" hypothesis, however, was first suggested in 1967 by J.S. Griffith,[21] a mathematician at Bedford College in London, based on earlier inactivation studies and without the knowledge of a scrapie-specific protein. Other investigators were also able to find prion protein in infectious preparations. Diringer et al. reported a similar 26 kDa protein and showed a correlation between the presence of fibrils and infectivity.[22]

Characterization of PrP 27-30 was then undertaken. The N-terminal sequence of PrP was determined from protein purified by size exclusion HPLC.[23] Using this sequence information, a cDNA library was made and then the full sequence of PrP was determined.[24] Antibodies to PrP peptides were prepared and immunoblotting indicated that PrP was present in normal as well as scrapie-infected hamster brains. However, the amount of PrP was much higher in scrapie cases. Similar levels of mRNA were found in the diseased and normal brains.[25] The mouse[26] and human[27, 28] PrP sequences were found to be highly homologous with hamster PrP and no other known protein (Fig. 1).

The open reading frame of PrP is 254 amino acids. The first 22 amino acids are a signal peptide that is cleaved during biosynthesis.[29, 30] After the signal peptide is a region that contains 5 octarepeats of sequence: PHGGGWGQ. Between residues 100 and 150 is a highly hydrophobic sequence that is very strongly conserved in all species of PrP. In naturally produced PrP, a glycophosphatidyl inositol (GPI) anchor is attached to Ser 231 and the remainder of the protein is cleaved as this anchor is added.[31] Asparagine residues are variably glycosylated at positions 181 and 197.[32] The cysteines at positions 179 and 214 form a disulfide linkage.[30] Overall PrP has a net positive charge of 18 at pH 7.4. The bulk of this positive charge resides in the N-terminal portion of the molecule.

PrP is found in a normal and disease-specific form

While PrP is found in normal and scrapie-infected hamster brains, these forms were found to be very different. The normal or cellular form of PrP was designated PrP^{C} and the scrapie-associated form was named PrP^{Sc}. No covalent difference has been found between the forms[33] that can explain their substantially different properties. Preparations of PrP^{C} are not infectious while preparations of PrP^{Sc} are. They also differ in their solubility, sedimentation, and resistance to degradation by enzymes. PrP^{C} is easily extracted by detergents and PrP^{Sc} is not. Ultracentrifugation can separate the forms because in the absence of denaturing treatment PrP^{Sc} is largely aggregated. A limited protease treatment will remove PrP^{C} from PrP^{Sc} preparations, as PrP^{C} is readily degraded by proteases and PrP^{Sc} is partially resistant to them. Proteinase K (PK), a serine protease, is typically used[34] although resistance to other enzymes such as trypsin and chymotrypsin is observed. Treating PrP^{Sc} with PK for one hour results in digestion limited to the first approximately 67 amino acids (cleavage around residue 90) which accounts for the lost 6-7 kDa.[23, 29] However, PrP^{Sc} will be fully digested after a 24 hour treatment. Without enzymatic treatment all PrP is primarily 33-35 kDa. A limited digestion of PrP^{Sc} preparations does not affect the infectivity, but seems to be necessary for PrP^{Sc} to form amyloid fibrils.[35]

PrP^{C} is expressed at very low levels in cells and PrP^{Sc} accumulates primarily in extracellular space of the brains of infected animals. The properties and quantity of PrP^{Sc} make it easier to purify, although trace amounts of RNA, DNA, heparin, etc. are found in all preparations. It is very difficult to obtain and purify PrP^{C} in even microgram amounts. PrP^{Sc} was purified and spectra were taken of it. Caughey and coworkers found by Fourier transform infrared (FTIR) spectroscopy that PrP^{Sc} had about 50% ß-sheet structure.[36] PrP^{C} was purified by Pan et al.[37] in enough quantity for FTIR spectroscopy. FTIR indicated substantial

a-helix and random coil secondary structure present in PrP^C. Covalent modifications between the two forms of PrP have not been found, suggesting that the only difference between the isoforms is a conformational change. This change may be sufficient to produce TSEs.

Genetic studies of PrP

The TSEs are notable in that they are infectious, sporadic, and genetic diseases. Sequencing PrP from individuals in families that have a history of TSEs has found a large number of single mutations that predict a TSE will occur. However, infectivity from all CJD brains is similar. Whether the CJD was sporadic, inherited, or due to infection does not seem to affect infectivity levels. No mutations in other proteins have been found to explain their propensity to acquire TSEs.

The gene for PrP (PRNP) is located on the short arm of chromosome 20 and the homologous region of mouse chromosome 2.[28, 38] Some of the 18 mutations that have been found associated with TSEs in humans to date are the following: Pro to Leu at codon 102 with GSS;[39, 40] Ala to Val at codon 117 with GSS;[41, 42] Asp to Asn at codon 178 with CJD and FFI;[3] and Glu to Lys at codon 200 with CJD.[43] A different mutation that accounts for some CJD is the insertion of several extra octarepeat units.[44, 45] Most of the diseases caused by these mutations in humans have been transmitted to animals.

How these mutations affect PrP^C and PrP^{Sc} or why so many varied mutations could end with the formation of PrP^{Sc} is unknown. Possibly, the mutations affect the folding or unfolding of PrP^C which aids in the formation of the disease specific form. Perhaps PrP^{Sc} aggregates are stabilized by the mutations. Mutations that cause TSEs is evidence that PrP plays an essential role

in the TSEs, but the argument can be made that PrP affects some other system or allows an individual to be susceptible to some ubiquitous agent.

Transgenic studies of PrP and the species barrier

The resistance to infection or extended incubation times that result when interspecies TSE transmissions are attempted are called species barriers. Mice inoculated with Chandler strain scrapie from other mice will get sick in approximately 120 days. Inoculating the same material into hamsters will cause scrapie in approximately 380 days.[46, 47] This increase in time is the species barrier. This 380 day incubation period will be reduced after several serial passages in hamsters. Hamster 263K scrapie will cause disease in hamsters in about 60 days, but does not cause disease in mice.[48] In this case the barrier is so large that total resistance is observed.

To learn how changing PrP affected the species barrier, transgenic (Tg) mice and hamsters were produced. The effect on the species barrier shed light on how PrP functions in the TSEs. First, the PRNP for the Syrian hamster was placed into mice. These mice would then produce hamster and mouse PrP (they differ at 16 residues). These Tg mice were viable and susceptible to infection by both mouse and hamster scrapie without any barrier.[49] The PrP^{Sc} produced in these Tg animals depended upon which species of scrapie was introduced. Inoculating Tg mice with hamster scrapie agent resulted in formation of only hamster PrP^{Sc} ($HaPrP^{Sc}$). Infected with mouse scrapie agent, only mouse PrP^{Sc} ($MoPrP^{Sc}$) was produced.[50] If the Tg mice had been inoculated with hamster scrapie, subsequent brain homogenates inoculated into normal hamsters resulted in no species barrier. This result strongly supports the hypothesis that the species barrier was due to the incompatibility of different species' PrP. Other Tg experiments have been done that are not as conclusive. Mice with the codon 102

Pro to Leu mutation that leads to GSS in humans spontaneously develop a neurodegenerative disease. However, the pathology is different from what is seen in humans and the disease is not transmissible.[50] Therefore, this mutation in mice may not cause a TSE. Mice that express many fold excess PrP also develop a neurodegenerative disease that may not be scrapie because it is also not transmissible.[51]

An important Tg experiment was done by Weissman and coworkers using mice with no functioning PrP gene or "knockout" mice.[52] These mice are viable and seem normal under laboratory conditions. However, recent studies of hippocampal tissue from these mice have indicated that long term potentiation abnormalities may be present.[53] These mice were inoculated with mouse scrapie to determine their incubation period. It was found that the "knockout" mice were not susceptible to scrapie, illustrating that PrP is a necessary component of scrapie pathology. Recently, experiments with mice expressing human and/or mouse PrP^C have shown that CJD is much more easily transmitted to the knockout mice expressing only human PrP^C.[54] CJD transmission to mice is hindered when only 5-10% of the total PrP^C expressed is of the mouse sequence. The authors state that this suggests a required species-specific non-PrP cofactor in the conversion process. However, a putative cofactor remains to be identified.

TSE strains

Strains of TSEs that have different pathologies exist within the same species. Many strains are stable and will maintain their distinct pathologies after indefinite serial passages. The existence of different strains of TSEs has been used as evidence that different virus strains are causing the diseases. The pathological differences include different patterns of spongiform degeneration, different areas of the brain being affected, and widely varying incubation times.

Most strains have resulted through interspecies TSE transmissions and different serial passage histories.

For example, 263K, which is a widely used strain of hamster scrapie, came from passage of sheep scrapie to mice[11] which became the Chandler strain. After the fifth serial passage of this mouse scrapie in Syrian golden hamsters, the 263K strain emerged.[12] The characteristics of this strain in hamsters does not vary after indefinite serial passages.

Biosynthesis of PrP^C and PrP^{Sc} in cell culture

The production of scrapie infectivity occurs in the brains of inoculated hamsters and other animals, but the simplest system that is known to produce infectivity is a neuroblastoma cell system. While the function of PrP^C is unknown, this system has been used to study the biosynthesis of PrP^C and PrP^{Sc}. Neurons express more PrP than other cells and also have the highest concentration of PrP mRNA. For this reason infected brains are used as the source of PrP^{Sc} and neural cells are used to study the biosynthesis.

Cell culture studies of PrP biosynthesis were made possible with the development of a chronically infected murine neuroblastoma cell line. After an initial treatment with mouse scrapie, these cells continuously produce PrP^{Sc} and infectivity. Pulse-chase metabolic labeling using ^{35}S-methionine followed by an immunoprecipitation with a PrP antibody was done.[55] ^{35}S-PrP^C was found two minutes after addition of the ^{35}S, but labeled PrP^{Sc} was not detected until one hour later. ^{35}S-PrP^C concentration was found to peak about two hours after labeling and it was degraded in the cell with a half-life of about four hours. ^{35}S-PrP^{Sc} continued to increase up to 45 hours later with half-maximal labeling at 6 hours. There was no evidence of degradation or turnover occurring. The distinction between PrP^C and PrP^{Sc} was made using PK digestion which showed

that PrP^C was the precursor of PrP^{Sc}. Only 3% of the total ^{35}S-PrP^C that was produced eventually became ^{35}S-PrP^{Sc}. Phosphatidylinositol-specific phospholipase C (PIPLC) cleaves the GPI anchor of PrP and releases it from the cell surface. The use of PIPLC on the neuroblastoma cells after metabolic labeling inhibited the conversion to PrP^{Sc}, suggesting the conversion occurs after PrP^C is brought to the cell surface.

What is the infectious agent in the TSEs?

One of the most puzzling questions concerning TSEs is the identity and function of the infectious agent associated with the diseases. The answer must account for the TSEs being infectious, genetic, and sporadic diseases, and also the species barriers and strains that exist.

The TSE infectious agent has the biological properties of a virus but its chemical properties are not typical of most viruses. Typical of a virus are the barriers to interspecies transmission and the strains that exist. However, the agent has been found to be resistant to chemical denaturants and treatments that destroy most normal viruses. After years of searching, no conventional agent or TSE-specific nucleic acid has been found. The infectious agent of the TSEs is certainly unconventional but many do not believe it is only protein, as such an infectious agent is totally unprecedented. The evidence for PrP^{Sc} being a necessary component of the infectious agent is strong.[56] As mentioned earlier, treatments that disrupt nucleic acids do not affect infectivity. PrP^{Sc} copurifies with infectivity and cannot be separated from it. Extensive protease treatments that degrade PrP^{Sc} eliminate infectivity. There is also a strong genetic linkage between specific PrP mutations and TSEs in humans. Finally, PrP knockout mice do not get scrapie. While this evidence ties PrP^{Sc} to infectivity, the involvement of potential cofactors that purify with PrP can not be ruled out.[57] These could

conceivably include any of the molecules found in PrP preparations such as small RNAs or DNAs, glycosoaminoglycans, or heparins, for example.[58-60] Proof of a protein only infectious agent or any other theory has not been possible. Up to now infectivity has only been created in an animal or cell culture and the purification always leaves the question of some minor contaminant that could be the agent. Absolute proof of the "protein only" theory requires de novo formation of infectivity from purified peptide.

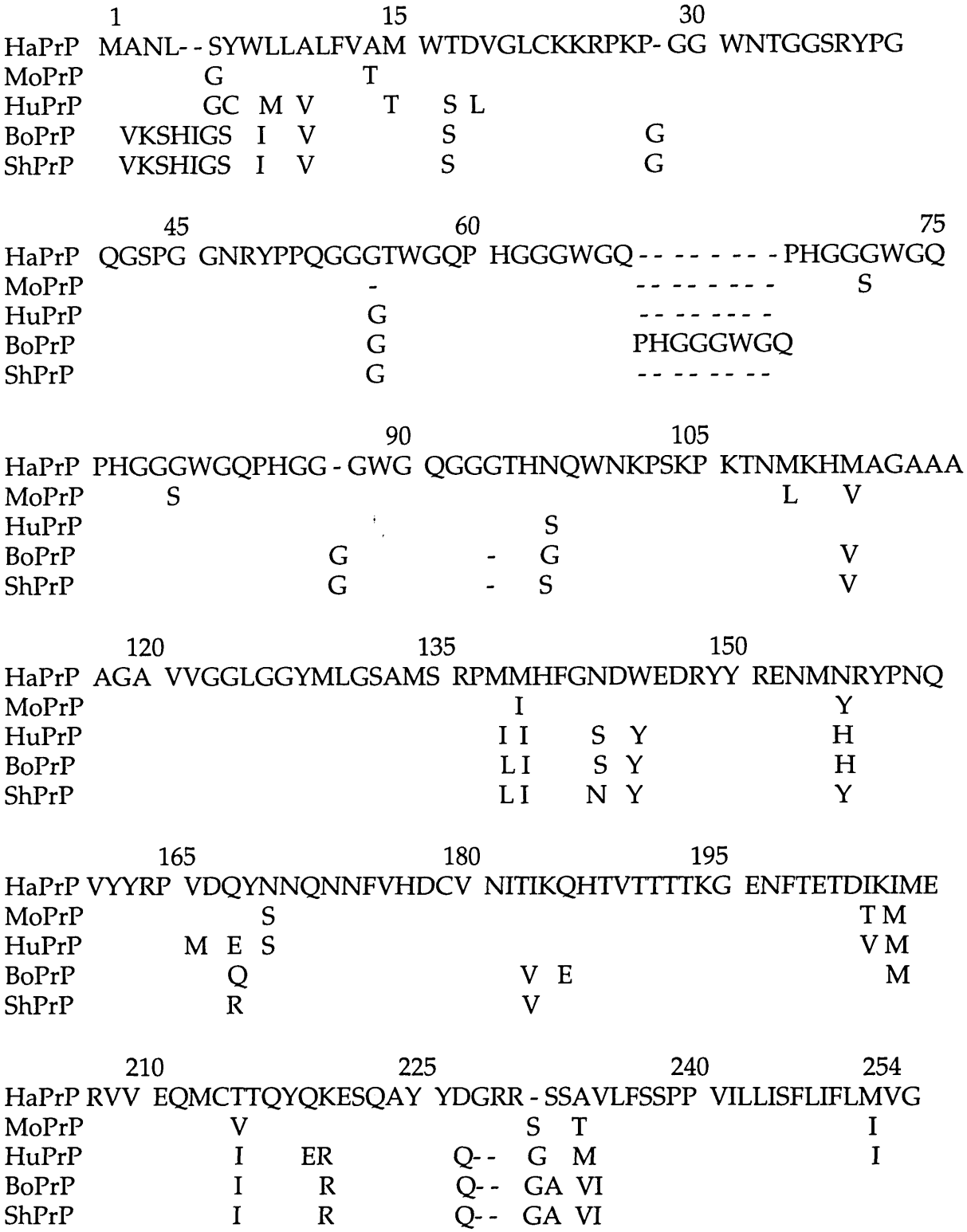
The protein only theory can explain all of the aspects of the TSEs described above. A large portion of this thesis explains how PrP^{Sc} could be the infectious agent of scrapie using results from a cell-free PrP^{C} conversion system.

A cell-free PrP^{C} conversion system

In the summer of 1993, a collaboration was initiated between the Lansbury lab at MIT and the Caughey lab at Rocky Mountain Laboratories (RML), an NIH laboratory in Hamilton, Montana. The purpose was to study the folding and unfolding of PrP^{Sc} and the conversion of PrP^{C} to PrP^{Sc}. Renaturation conditions for PrP^{Sc} were discovered and the conversion of PrP^{C} to the protease-resistant form was demonstrated soon after. This conversion system made possible many informative experiments that will be covered in the following chapters. Chapter 2 deals with the renaturation of PrP^{Sc} and the conversion of labeled PrP^{C} to a protease-resistant form. Chapter 3 is about the initial mechanistic studies undertaken to determine how the conversion of PrP^{C} to the protease-resistant form takes place. Chapter 4 is about the study of the TSE species barriers using the cell-free system. Finally, chapter 5 covers the use of the cell-free system to mimic the passage of strain-specific information that is seen *in vivo.*

Figure 1. The sequence of prion protein in various mammals. HaPrP, hamster PrP; MoPrP, mouse PrP; HuPrP, human PrP; BoPrP, bovine PrP; ShPrP, ovine (sheep) PrP. Asn 181 and Asn 197 are glycosylated. A disulfide exists between Cys 179 and Cys 214. Treatment of PrP^{Sc} with PK results in cleavage around residue 90.

Fig. 1


1 15 30
HaPrP MANL--SYWLLALFVAM WTDVGLCKKRPKP-GG WNTGGSRYPG
MoPrP G T
HuPrP GC M V T S L
BoPrP VKSHIGS I V S G
ShPrP VKSHIGS I V S G
45 60 75
HaPrP QGSPG GNRYPPQGGGTWGQP HGGGWGQ-- -- - -- -PHGGGWGQ
MoPrP - -- -- - -- - S
HuPrP G -- -- -- - -
BoPrP G PHGGGWGQ
ShPrP G -- -- -- --
90 105
HaPrP PHGGGWGQPHGG-GWG QGGGTHNQWNKPSKP KTNMKHMAGAAA
MoPrP S L V
HuPrP S
BoPrP G - G V
ShPrP G - S V
120 135 150
HaPrP AGA VVGGLGGYMLGSAMS RPMMHFGNDWEDRYY RENMNRYPNQ
MoPrP I Y
HuPrP I I S Y H
BoPrP L I S Y H
ShPrP L I N Y Y
165 180 195
HaPrP VYYRP VDQYNNQNNFVHDCV NITIKQHTVTTTTKG ENFTETDIKIME
MoPrP S T M
HuPrP M E S V M
BoPrP Q V E M
ShPrP R V
210 225 240 254
HaPrP RVV EQMCTTQYQKESQAY YDGRR-SSAVLFSSPP VILLISFLIFLMVG
MoPrP V S T I
HuPrP I ER Q-- G M I
BoPrP I R Q-- GA VI
ShPrP I R Q-- GA VI

REFERENCES FOR CHAPTER 1

1. Kimberlin, R.H. & Walker, C.A. *Antimicrobial Agents and Chemotherapy* **30**, 409-413 (1986).
2. Kitamoto, T. & Tateishi, J. *Am. J. Pathol.* **131**, 435-443 (1988).
3. Goldfarb, L.G., Peterson, R.B., Tabaton, M., Brown, P., LeBlanc, A.C., Montagna, P., Cortelli, P., Julien, J., Vital, C., Pendelbury, W.W., Halta, M., Willis, P.R., Hauw, J.J., McKeever, P.E., Monari, L., Schrank, B., Swergold, G.D., Autillo-Gambetti, L., Gajdusek, D.C., Lugaresi, E. & Gambetti, P. *Science* **258**, 806-808 (1992).
4. Tateishi, J., Brown, P., Kitamoto, T., Hoque, Z.M., Roos, R., Wollman, R., Cervenakova, L. & Gajdusek, D.C. *Nature* **376**, 434-435 (1995).
5. Gajdusek, D.C. *Science* **197**, 943-960 (1977).
6. Sigurdarson, B. *Br. Vet. J.* **110**, 341 (1954).
7. Hadlow, W.J. *Lancet* **1959-II**, 289 (1959).
8. Gajdusek, D.C., Gibbs, C.J. & Alpers, M. *Nature* **209**, 794-796 (1966).
9. Gibbs, C.J. *Science* **161**, 388 (1968).
10. Gibbs, C.J. & Gajdusek, D.C. *Science* **182**, 67 (1973).
11. Chandler, R.L. *Lancet* **1961-I**, 1378 (1961).
12. Kimberlin, R.H. & Walker, C.A. *J. Gen. Virol.* **34**, 295-304 (1977).
13. Marsh, R.F. & Kimberlin, R.H. *J. Infect. Disease* **131**, 104-110 (1975).
14. Prusiner, S.B., Groth, D.F., Cochran, S.P., Masiarz, F.R., McKinley, M.P. & Martinez, H.M. *Biochem.* **19**, 4883-4891 (1980).
15. Stamp, J.T., Brotherston, J.G., Zlotnik, I., Mackay, J.M.K. & Smith, W. *J. Comp. Path.* **69**, 268 (1959).
16. Alper, T., Haig, D.A. & Clarke, M.C. *Biochem. Biophys. Res. Comm.* **22**, 278-284 (1966).
17. Merz, P.A., somerville, R.A., Wisniewski, H.M. & Iqbal, K. *Acta Neuropathol. (Berl.)* **54**, 63 (1981).
18. Merz, P.A., Rohwer, R.G., Kascsak, R., Wisniewski, H.M., Somerville, R.A., Gibbs, C.J., Jr. & Gajdusek, D.C. *Science* **437** (1984).
19. Prusiner, S.B., McKinley, M.P., Bowman, K.A., Bolton, D.C., Bendheim, P.E., Groth, D.F. & Glenner, G.G. *Cell* **35**, 349-358 (1983).
20. Bolton, D.C., McKinley, M.P. & Prusiner, S.B. *Science* **218**, 1309-1311 (1982).
21. Griffith, J.S. *Nature* **215**, 1043-1044 (1967).
22. Diringer, H., Gelderblom, H., Hilmert, H., Özel, M., Edelbluth, C. & Kimberlin, R.H. *Nature* **306**, 476-478 (1983).
23. Prusiner, S.B., Groth, D.F., Bolton, D.C., Kent, S.B. & Hood, L.E. *Cell* **38**, 127-134 (1984).
24. Oesch, B., Westaway, D., Wälchli, M., McKinley, M.P., Kent, S.B.H., Aebersold, R., Barry, R.A., Tempst, P., Teplow, B.B., Hood, L.E., Prusiner, S.B. & Weissmann, C. *Cell* **40**, 735-746 (1985).

25. Chesebro, B., Race, R., Wehrly, K., Nishio, J., Bloom, M., Lechner, D., Bergstrom, S., Robbins, K., Mayer, L., Keith, J.M., Garon, C. & Haase, A. *Nature* **315**, 331-333 (1985).
26. Locht, C., Chesebro, B., Race, R. & Keith, J.M. *Proc. Natl. Acad. Sci. USA* **83**, 6372-6376 (1986).
27. Kretzschmar, H.A., Stowring, L.E., Westaway, D., Stubblebine, W.H., Prusiner, S.B. & DeArmond, S.J. *DNA* **5**, 315-324 (1986).
28. Liao, Y.-C.J., Lebo, R.V., Clawson, G.A. & Smuckler, E.A. *Science* **233**, 364-367 (1986).
29. Hope, J., Morton, L.J.D., Farquhar, C.F., Multhaup, G., Beyreuther, K. & Kimberlin, R.H. *The EMBO J.* **5**, 2591-2597 (1986).
30. Turk, E., Teplow, S.B., Hood, L.E. & Prusiner, S.B. *Eur. J. Biochem.* **176**, 21-30 (1988).
31. Stahl, N., Borchelt, D.R., Hsiao, K.K. & Prusiner, S.B. *Cell* **51**, 229-240 (1987).
32. Haraguichi, T., Fisher, S., Olofsson, S., Endo, T., Groth, D., Tarentino, A., Borchelt, D., Teplow, D., Hood, L., Burlingame, A., Lycke, E., Kobata, A. & Prusiner, S.B. *Arch. Biochem. Biophys.* **274**, 1-13 (1989).
33. Stahl, N., Baldwin, M.A., Teplow, D.B., Hood, L., Gibson, B.W., Burlingame, A.L. & Prusiner, S.B. *Biochem.* **32**, 1991-2001 (1993).
34. Meyer, R.K., McKinley, M.P., Bowman, K.A., Braunfeld, M.B., Barry, R.A. & Prusiner, S.B. *Proc. Natl. Acad. Sci. USA* **83**, 2310-2314 (1986).
35. McKinley, M.P., Meyer, R.K., Kenaga, L., Rahbar, F., Cotter, R., Serban, A. & Prusiner, S.B. *J. Virology* **65**, 1340-1351 (1991).
36. Caughey, B.W., Dong, A., Bhat, K.S., Ernst, D., Hayes, S.F. & Caughey, W.S. *Biochemistry* **30**, 7672-7680 (1991).
37. Pan, K.-M., Baldwin, M., Nguyen, J., Gasset, M., Serban, A., Groth, D., Mehlhorn, I., Huang, Z., Fletterick, R.J., Cohen, F.E. & Prusiner, S.B. *Proc. Natl. Acad. Sci. USA* **90**, 10962-10966 (1993).
38. Sparkes, R.S., Simon, M., Cohn, V.H., Fournier, R.E.K., Lem, J., Klisak, I., Heinzmann, C., Blatt, C., Lucero, M., Mohandas, T., DeArmond, S.J., Westaway, D., Prusiner, S.B. & Weiner, L.P. *Proc. Natl. Acad. Sci. USA* **83**, 7358-7362 (1986).
39. Hsiao, K., Baker, H.F., Crow, T.J., Poulter, M., Owen, F., Terwilliger, J.D., Westaway, D., Ott, J. & Prusiner, S.B. *Nature* **338**, 342-345 (1989).
40. Doh-ura, K., Tateishi, J., Sasaki, H., Kitamoto, T. & Sakaki, Y. *Biochem. Biophys. Res. Comm.* **163**, 974-979 (1989).
41. Nochlin, D., Sumi, S.M., Bird, T.D., Snow, A.D., Leventhal, C.M., Beyreuther, K. & Masters, C.L. *Neurology* **39**, 910-918 (1989).
42. Hsiao, K.K., Cass, C., Schellenberg, G.D., Bird, T., Devine-Gage, E., Wisniewski, H. & Prusiner, S.B. *Neurol.* **41**, 681-684 (1991).
43. Goldfarb, L., Korczyn, A., Brown, P., Chapman, J. & Gajdusek, D.C. *Lancet* **2**, 637-8 (1990).
44. Owen, F., Poulter, M., Shah, T., Collinge, J., Lofthouse, R., Baker, H., Ridley, R., McVey, J. & Vrow, T.J. *Molec. Brain. Res.* **7**, 273-276 (1990).
45. Collinge, J., Brown, J., Hardy, M., Mullan, M.J., Rossor, M.N., Baker, H.F.,

Crow, T.J., Lofthouse, R., Poulter, M., Ridley, R.M., Owen, F., Bennett, C., Dunn, G., Harding, A.E., Quinn, N., Doshi, R., Roberts, G.W., Honavar, M., Janota, I. & Lantos, P.L. *Brain* **115**, 687-710 (1992).
46. Kimberlin, R.H. & Walker, C.A. *J. Gen. Virol.* **39**, 487-496 (1978).
47. Kimberlin, R.H., Cole, S. & Walker, C.A. *J. Gen. Virol.* **68**, 1875-1881 (1987).
48. Kimberlin, R.H., Walker, C.A. & Fraser, H. *J. Gen. Virol.* **70**, 2017-2025 (1989).
49. Prusiner, S.B., Scott, M., Foster, D., Pan, K.-M., Groth, D., Mirenda, C., Torchia, M., Yang, S.-L., Serban, D., Carlson, G.A., Hoppe, P.C., Westaway, D. & DeArmond, S.J. *Cell* **63**, 673-686 (1990).
50. Scott, M., Foster, D., Mirenda, C., Serban, D., Coufal, F., Walchli, M., Torchia, M., Groth, D., Carlson, G., DeArmond, S.J., Westaway, D. & Prusiner, S.B. *Cell* **59**, 847-857 (1989).
51. Westaway, D., DeArmond, S.J., Cayetano-Canlas, J., Groth, D., Foster, D., Yang, S.-L., Torchia, M., Carlson, G.A. & Prusiner, S.B. *Cell* **76**, 117-129 (1994).
52. Büeler, H., Fischer, M., Lang, Y., Bluethmann, H., Lipp, H.-P., DeArmond, S.J., Prusiner, S.B., Aguet, M. & Weissmann, C. *Nature* **356**, 577-582 (1992).
53. Collinge, J., Whittington, M.A., Sidle, K., Smith, C.J., Palmer, M.S., Clarke, A.R. & Jefferys, J. *Nature* **370**, 295-297 (1994).
54. Telling, G.C., Scott, M., Mastrianni, J., Gabizon, R., Torchia, M., Cohen, F.E., DeArmond, S.J. & Prusiner, S.B. *Cell* **83**, 79-90 (1995).
55. Caughey, B. & Raymond, G.J. *J. Biol. Chem.* **266**, 18217-18223 (1991).
56. Prusiner, S.B. *Science* **252**, 1515-1522 (1991).
57. Caughey, B. & Raymond, G.J. *J. Virol.* **67**, 643-650 (1993).
58. Snow, A.D., Wight, T.N., Nochlin, D., Koike, Y., Kimata, K., Dearmond, S.J. & Prusiner, S.B. *Lab. Invest.* **63**, 601-611 (1990).
59. Riesner, D. *Sem. Virol.* **2**, 215-226 (1991).
60. Aiken, J.M. & Marsh, R.F. *Microbiol. Rev.* **54**, 242-246 (1990).

Chapter 2
The cell-free conversion of hamster PrP^C to the protease-resistant form

Formation of protease-resistant PrP is known to occur in scrapie-infected animals and chronically scrapie-infected neuroblastoma cells. Previously, these cells were the simplest system capable of converting PrP to the protease-resistant form. To study the mechanism of the conversion of PrP^C to PrP^{Sc}, a simpler system was required that does not have the myriad of variables inherent in a biological system. An *in vivo* system is useful in studying the biosynthesis of PrP, but the intention was to concentrate on the chemistry of the protein itself as much as possible. Development of a cell-free conversion system was the first goal of the work at RML.

The renaturation of PrP^{Sc} as visualized by antibodies to PrP epitopes

It is assumed throughout this study that an undefined level of unfolding must occur for a normally protease-resistant peptide sequence of PrP^{Sc} to become protease-sensitive. Conditions that allowed denaturation of PrP^{Sc} (as judged by loss of resistance to PK) and renaturation (as seen by the return of PK resistance) after the denaturant was diluted were found. PrP^{Sc} at a concentration of about one μg/μL was first treated in 2.5-3 M guanidine-HCl (GdnHCl),[1] a chaotropic salt. To get an accurate time zero point, a sample of PrP^{Sc} in the presence of 2.5 or 3 M GdnHCl was treated with PK to assay its resistance before any renaturation could begin. PK is a very hardy enzyme that functions in extremes of temperature, pH, and denaturant. The PrP was visualized through the use of

sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) and then an immunoblot protocol which initially used a polyclonal antibody to PrP peptide 90-104. Immunoblotting with the PrP 90-104 antibody, there was trace or no PK resistance in the presence of 3 M GdnHCl. The PrP^{Sc} in 2.5 or 3 M GdnHCl was then diluted to 0.75 M with a Tris-HCl, NaCl (TN) solution. This mixture was allowed to incubate at 37° C for between 2 minutes and 2 days and then the PK resistance was determined. Some return of PK resistance in the PrP^{Sc} was seen in as little as 2 minutes before the addition of PK and more was seen after 2 days. Of course the PK needs time to hydrolyze the PrP and renaturation in the 2 minute samples can be occurring as this happens, which emphasizes the need for the time zero point.

To better define the effects of various concentrations of GdnHCl on the protease-resistance of PrP^{Sc}, multiple antibodies were used in immunoblot analyses of PrP^{Sc} subjected to these treatments. Also, to simplify the analysis of PrP bands seen in this type of experiment, PrP^{Sc} was treated with peptide N glycosidase F (PNGase F) immediately prior to SDS-PAGE. PNGase F removes N-linked sugars which account for some of the band multiplicity of PrP.

After PK and PNGase F treatment of PrP^{Sc} without any denaturant present, a predominant 19 kDa band (Fig. 2.1, marked by the solid arrowhead) was detected using antisera to PrP residues 90-104, 106-115 (3F4), 143-156, and 217-232 (lane 2). As expected, residues 90-232 were resistant to PK. Without PNGase F treatment, additional larger bands were detected, which represent glycosylated forms of the 19 kDa polypeptide (lane 8). When PrP^{Sc} was digested with PK in the presence of 2.5 or 3 M GdnHCl, the detection of the 19 kDa, PNGase F-treated band by all three antibodies was greatly reduced (lane 3). Instead, a prominant 16 kDa band (open arrowheads) was observed using the antisera to PrP 143-156 and 217-232, but not with the antisera to PrP 90-104 or

106-115 (lane 3). Similarly, in the samples not treated with PNGase F (lane 9), little PrP was detected with the 90-104 or 106-115 antisera (lane 9, top panels), but the 143-156 and 217-232 antiersa detected sets of bands that were ~3 kDa smaller than those generated by PK treatment in the absence of GdnHCl (compare lanes 8 and 9, lower panels). These results showed that the GdnHCl treatment made an additional ~3 kDa of the PrP^{Sc} polypeptide backbone sensitive to PK. Because the detection by 90-104 and 106-115 antiersa was reduced much more than the detection by the 217-232 antibody, most of the additional mass was lost from the N-terminus. The calculated molecular mass of residues 90-115 is 3210, so the removal of these residues could alone could account for the additional mobility in SDS-PAGE. However, there was a partial loss of detection by the 217-232 antibody (compare lanes 8 and 9, lower panel) indicating that part of this epitope was also removed by PK. Thus, the major effect of treatment with 2.5-3 M GdnHCl was to denature PrP^{Sc} from residue 90 to the vicinity of residue 115, with a less pronounced unfolding of the 217-232 epitope at the C-terminus.

The recovery of PK-resistance upon dilution of the GdnHCl and incubation is also shown in Fig. 2.1 [compare lanes 3 with 4 and 5 with 6 (deglycosylated samples) and lanes 9 with 10 and 11 with 12 (glycosylated samples)]. The antisera to PrP residues 90-104 and 106-115 indicated an increase in the 19 kDa unglycosylated band (solid arrowhead) and an increase in the glycosylated bands as well. The antibodies to PrP 143-156 and 217-232 indicated a decrease in the 16 kDa unglycosylated band and an increase in the 19 kDa unglysocylated band. Analysis of the glycosylated species also showed an upward shift of ~3 kDa in the PK-resistant bands, giving a set of bands similar in size to the control samples that were treated with PK in the absence of GdnHCl. The extent of renaturation of all of these epitopes was greater after the 2.5 M

GdnHCl treatment than the 3 M treatment. The recovery of the PK-resistance of all epitopes tested indicated that the denaturing effect of 2.5 and 3 M GdnHCl on PrP^{Sc} was partially reversible.

This system for renaturation appeared to be working well, so a lot of effort further altering variables was not done. Incubation time and temperature were tested and some other denaturants were used. Using higher temperatures was counter productive because irreversible denaturation occurred in 0.75 M GdnHCl at 50° C. No benefit was found in extending the time for refolding to occur. The maximum refolding would occur in two days or less and was usually 75% or higher based on comparisons with undenatured, PK-treated samples. Very little renaturation occurred from 3.5 M GdnHCl and denaturation in 4 M or higher GdnHCl at 37° C was permanent (Fig. 2.2). A map of PrP (Fig. 2.3) summarizes the PK cleavage that occurred in the presence of different concentrations of GdnHCl. 2 M or higher guanidine thiocyanate (GdnSCN) at 37° C produced irreversible denaturation but the effect of 1 M was reversible (Fig. 2.4). Note that with all antibodies the effect of 1 M GdnSCN on PrP^{Sc} was almost identical to 3 M GdnHCl. 25% (v/v) or higher hexafluoroisopropanol (HFIP) also irreversibly denatured PrP^{Sc}. The use of GdnSCN or other denaturing conditions was not pursued further.

Cell-free conversion of hamster PrP^{C} to the protease-resistant form

Some of the steps in the process of refolding portions of PrP^{Sc} to the PK-resistant form may be occurring during the conversion of PrP^{C} to PrP^{Sc}. For this reason, conditions that allowed refolding of PrP^{Sc} were used to convert PrP^{C} to the protease-resistant form. A source of ^{35}S labeled PrP^{C} was available from high expressing cell lines developed at RML. ^{35}S-PrP^{C} was purified by immunoprecipitation using a PrP antibody on protein A sepharose beads. The

^{35}S-PrP^{C} was removed from the beads by eluting it in 3 M GdnHCl. The seed or nucleus for conversion was 2.5-3 M GdnHCl pretreated PrP^{Sc} that had been prepared from hamster brains earlier for use in renaturation experiments. The PrP^{Sc} and ^{35}S-PrP^{C} were mixed and diluted to 0.75 M GdnHCl using TN solution and incubated for two days at 37° C. The concentration of PrP^{Sc} in the conversion reaction was about 0.25 μg/μL with the ^{35}S-PrP^{C} concentration about 50 fold less. PK was added and the products were run on SDS-PAGE and the ^{35}S visualized using fluorography. This procedure resulted in PK-resistant ^{35}S-PrP reduced by about 7 kDa compared to the labeled starting material. This reduction would be expected after treating PrP^{Sc} with PK.

PrP dimers and PrP without a GPI anchor converted to the resistant form

For this initial study, two different forms of PrP^{C} were used. One was recombinant hamster PrP^{C} expressed in mouse neuroblastoma cells. This was composed of monomeric PrP of 30-40 kDa and a 60 kDa dimer (Fig. 2.5**a**). The monomer form varied over this range because of different levels of glycosylation.[2, 3] After incubation with PrP^{Sc} and treatment with PK, the predominant band was at 24 kDa, but there was some higher molecular weight material produced. A 24 kDa band also predominates when scrapie-infected cells are treated with PK. Ultracentrifugation was used to separate the monomeric and dimeric forms of this PrP^{C} construct. The separated forms were then used in similar conversion reactions (Fig. 2.5**b**). Both converted to resistant forms during incubation and different migrations were observed in the predominant bands after PK treatment. The variation might have been due to covalent differences between monomer and dimer affecting the action of PK.

The other PrP^{C} construct used was a hamster PrP with a stop codon at position 231 which usually has the GPI anchor attached. Therefore this construct

lacked residue 231 and the GPI anchor. In normal cell processing, amino acids 232 through 254 are cleaved as the GPI anchor is added. The lack of residue 231 and GPI anchor in this construct are the only differences between it and wild type hamster PrP. The predominant 24 kDa species of this PrP^C was unglycosylated with a smaller amount of a 28 kDa monoglycosylated form (Fig. 2.5c). When this PrP^C was incubated with PrP^{Sc} and PK treated, the predominant band was at 17 kDa, 7 kDa less than the starting material. Again this was expected after limited protease digestion to about residue 90. As had been seen in other scrapie-infected cell culture systems, the GPI anchor was not required for protease-resistance and a similar 17 kDa product has resulted from scrapie infection in cell culture.[4]

Controls of the cell-free conversion

Controls were needed to show that there was specificity in the conversion of PrP^C to the PK-resistant form. The reaction was done with no PrP^{Sc} present and no conversion was seen (Fig 2.5). The reaction was treated with PK after a two minute incubation instead of two days. Two minutes was not enough time to see any conversion which demonstrated that the conversion occurred after mixing and longer incubation. PrP^{Sc} was added that had been pretreated with 0, 3, and 6 M GdnHCl. 3 M treated PrP^{Sc} gave more conversion than 0 M, and 6 M gave no detectable conversion. The exact GdnHCl pretreatment that maximized the conversion varied between 2.5 and 3 M GdnHCl depending on the PrP^{Sc} preparation used. Fig. 2.6 shows conversion after different GdnHCl and GdnSCN pretreatments of the PrP^{Sc}. Here the 2.5 M GdnHCl pretreatment allowed more efficient conversion than 0 or 3 M GdnHCl pretreatment. 3.5 M GdnHCl pretreatment yielded barely detectable conversion and 4 M prevented all conversion. 1 M GdnSCN pretreatment allowed a small amount of

conversion, but 2 M prevented all conversion. The denaturing conditions that prevented recovery of the PK-resistance of PrP^{Sc} also eliminated its ability to convert PrP^{C} to the resistant form. Finally, these controls showed that a PrP^{Sc} preparation with some native structure was required for the conversion and that it occurred during the two day incubation period.

Another control was done to show that ^{35}S-PrP^{C} was the only labeled protein from the cells that would become PK-resistant after incubation with PrP^{Sc}. This control was accomplished by adding a set number of ^{35}S counts per minute (cpm) of lysate proteins while otherwise running the reaction as usual. The ratio of cpm of PrP^{C} versus lysate was about 1:15. Even in the presence of the lysate proteins, the only specific band seen was from a protein whose migration was identical to protease-resistant PrP (Fig. 2.7). This control shows that it is probably not a general occurrence for proteins to gain protease resistance after incubation with PrP^{Sc}. It would have been unexpected for other proteins to become PK-resistant as a general occurrence, as many proteins are not becoming resistant in scrapie-infected animals. The lysate is a mixture of many proteins and a weakness of this control is that if one specific other protein could become resistant it might be present with too few counts to be detected after PK treatment.

The final controls were to show that the protease-resistance seen was not limited to PK, and that the conversion was specific to the addition of a PrP^{Sc} preparation to the reaction and not any amyloid. To check the resistance to other proteases, trypsin and chymotrypsin were simply substituted for PK. PK-resistant ^{35}S-PrP was also trypsin and chymotrypsin resistant (Fig. 2.8). The ladder of bands seen after trypsin digest was the result of cleavages after Lys or Arg. These cuts probably occurred in the more protease sensitive N-terminal portion of the molecule. ß-amyloid from an Alzheimer's patient was pretreated

with 0 M and 3 M GdnHCl and then substituted for PrP^{Sc} in the reaction. Incubation with ß-amyloid did not result in any protease resistance (Fig. 2.8, lanes 7-8). More than just amyloid structure is required for the conversion to occur. An even better control than ß-amyloid is to use a different species PrP^{Sc} and this experiment is discussed extensively in chapter 4.

Conversion experiments with tunicamycin treated PrP^{C}s

Because the complexity of the pattern of ^{35}S-labeled PK-resistant bands generated was due to variation in the N-linked glycosylation of the PrP^{C} precursor, the conversion experiment was done with PrP^{C} from cells treated with tunicamycin, which prevents N-linked glycosylation. The Asn residues at positions 181 and 197 are glycosylated during biosynthesis. The addition of tunicamycin to cells expressing recombinant hamster PrP^{C} after labeling produced bands at 25 and about 50 kDa in SDS-PAGE, (Fig. 2.9) which represent unglycosylated monomeric and dimeric forms. In the conversion reaction, the tunicamycin-treated PrP yielded a major band at 19 kDa after treatment with PK. This band migrated in SDS-PAGE similarly to the predominant form of the GPI^{-} construct, which is also unglycosylated, after treatment with PK. The difference was probably due to the presence of the GPI anchor affecting migration. This is evidence that glycosylation was not required to achieve protease-resistance in this cell-free system.

Limitations of this cell-free conversion system

To show that this system is generating ^{35}S-PrP^{Sc}, the production of new infectivity needs to be demonstrated. Unfortunately, that was not possible using this system because of the vast amount of infectivity present when the PrP^{Sc} was added. Bioassays cannot measure infectivity precisely and to see a significant

difference between samples, there must be at least 100 or preferably 1000 fold difference in titer. This reaction has an approximate 50 fold excess of PrP^{Sc} to begin with, and any new infectivity would be insignificant.

The major protein ingredients in this reaction were PrP^{Sc}, ^{35}S-PrP^{C}, and the antibody used to immunoprecipitate it. The presence of this antibody was controlled for by the experiment showing no conversion without added PrP^{Sc}. The PrP^{Sc} preparations used have impurities that can not be controlled for which can include DNAs, RNAs, glycosaminoglycans (GAGs), etc. If a very minor component of the PrP^{Sc} preparation is a cofactor in the conversion as has been suggested,[5, 6] these experiments can not rule that out.

What this in vitro *conversion system means*

The initial studies of this cell-free conversion system were published in August, 1994.[7] It was stated in the manuscript that these results did not prove a "protein-only" hypothesis for TSEs, but press accounts of this paper overstated the results. This study did show that barring contaminants in the PrP^{Sc} preparation as potential cofactors, direct PrP^{C}-PrP^{Sc} interactions are required to convert PrP^{c} to the protease-resistant form. Little mechanistic detail was learned from the study, but the system can be used for that purpose (see chapter 3).

The value of a cell-free conversion system for PrP^{C} had already been recognized. Papers published by Prusiner and coworkers explain their frustrated attempts to convert PrP^{C} *in vitro*. In a 1992 attempt,[8] they mixed $MoPrP^{Sc}$ with recombinant mouse/hamster PrP^{C} that was converted to a resistant form in their infected cells. They used ^{35}S-PrP^{C} or a specific antibody to their recombinant PrP to detect new PK resistance after incubation at 37° C. They achieved no increase in protease-resistance, but it is difficult to tell what the concentration of any of the components was. Concentration is important in this system and too low a

PrP^{Sc} concentration could explain the failure. In a 1993 effort,[9] researchers denatured PrP^{Sc} and then unsuccessfully tried to recover infectivity. Their typical treatment was 6 M GdnSCN, which is quite harsh compared to the conditions reported here. Recently, the Prusiner lab was able to reproduce the *in vitro* conversion described in this chapter[10]. Depending on the actual mechanism of renaturation, a seed of native PrP^{Sc} structure might be required for recovery of infectivity. PrP^{C} treated with as much as 6 M GdnHCl was converted to the protease-resistant form with the conversion system presented here, and the seed of PrP^{Sc} with PK-resistant structure was crucial to the conversion.

This system presented an opportunity to study the conversion of PrP^{C} to a PrP^{Sc}-like form without all the complications of an *in vivo* system and was evidence that the conversion may be solely due to direct PrP-PrP interactions. This direct interaction is the essence of "protein-only" theories which state that the infectious agent is only PrP^{Sc} that replicates itself in a new host. The system allows a detailed look at the mechanism of the conversion and other biological questions of the TSEs to be addressed.

Figure 2.1 Partial reversible unfolding of PrP^{Sc} in 2.5 and 3 M GdnHCl. Aliquots of PrP^{Sc} were treated with PK in the designated concentration of GdnHCl (renaturation = 0) or after four fold dilution and incubation for 1 d (renaturation = 1 d) and further dilution prior to PK treatment. Control samples were not treated with PK (lanes 1 and 7) or treated with PK without GdnHCl treatment (lanes 2 and 8). The two columns of panels represent identically treated sets of samples except for the use of PNGase F to deglycosylate the PrP samples immediately prior to SDS-PAGE in the left column (lanes 1-6).
In lanes 3 and 5 in the a143-156 panel, the faint 27-30 kDa bands indicated that the PNGase F was not always completely effective. Molecular weight markers are indicated at the right in kilodaltons.

Figure 2.2 At high GdnHCl concentration, unfolding of PrP^{Sc} was not reversible. Multiple antibody analysis of GdnHCl- and PK-treated PrP^{Sc} was performed as described for Fig. 2.1 except that the designated (higher) GdnHCl concentrations were used in the initial unfolding pretreatment. The "renatured" samples were diluted to 0.75 M GdnHCl and incubated for the designated time. The amount of

PK-resistance seen after 2 minutes was not always as much as shown. Control PrP^{Sc} in lanes 1 and 2 was treated with PK (+) or not treated (-) without prior exposure to GdnHCl. Molecular weight markers are indicated at the left in kilodaltons.

Figure 2.3 Schematic depiction of PrP sequence, indicating PK-resistance of PrP^{Sc} under various denaturing and renatured conditions. The bars at the top represent PrP and the epitopes of the antibodies used in this study. The PK-resistant regions are approximated based on a combination of their reactivities with the antibodies and their sizes on the SDS-PAGE gels shown in Figs. 2.1 and 2.2. Where the termini are approximate, the borders are shown with jagged lines. With no GdnHCl present, PK cleaves PrP^{Sc} to about residue 90. In 3 M GdnHCl, PK eliminated most of the signal by the 90-104 and 106-115 antibodies, so the majority of N-terminal cleavage was at least to 115. Based on the loss of the 217-232 antibody signal, some proteolysis into its epitope at the C-terminus also occurred. Upon treatment by PK in 3.5 M GdnHCl, and also after attempted renaturation by dilution to 0.75 M GdnHCl, a small amount of PrP was detected by the 143-156 and 217-232 antibodies, both reduced in mass by ~3 kDa from normal PrP. This portion of PrP indicated by the hatched area in 3.5 M GdnHCl and the solid area in 3 M GdnHCl seemed to be the most resistant to denaturation (the 16 kDa protease-resistant peptide core). 4 M GdnHCl irreversibly eliminated all detectable PrP after PK treatment.

Figure 2.4 Partial unfolding of PrP^{Sc} was reversible from 1 M, but not ≥2 M GdnSCN. GdnSCN was substituted for GdnHCl in the unfolding/refolding procedures described in Fig. 2.1. The PK treatment was omitted in lane 1 (only). Note that 2 M or higher GdnSCN inhibited the ability of PK to digest PrP^{Sc} (lanes 6, 9, and 12). To renature, GdnSCN was diluted to 0.75 M and the samples incubated for 2' or 2 days before further dilution and treatment with PK. Note that after dilution of GdnSCN from 2, 3, or 4 M, PK was able to digest all PrP and no resistant material was seen after 2 days of incubation. Molecular weight markers are indicated at the left in kilodaltons.

Figure 2.5 Conversion of $^{35}S\text{-}PrP^{C}$ to PK-resistant forms in the presence of PrP^{Sc}. **a**, SDS-PAGE fluorography of $^{35}S\text{-}PrP^{C}$ with or without PrP^{Sc}. The $^{35}S\text{-}PrP^{C}$ was recombinant hamster PrP expressed in mouse neuroblastoma cells. Lanes 2-10 contained 7 times more ^{35}S PrP^{C} than the non PK treated lane 1. GdnHCl pretreatment refers to the concentration of GdnHCl the PrP^{Sc} was treated in before mixing with the $^{35}S\text{-}PrP^{C}$. Lanes 11 and 12 were immunoblots of PK-treated PrP^{Sc} from neuroblastoma cells (NB) and scrapie-infected hamster brain (Br). Lane 13 was an immunoblot of non PK-treated PrP^{Sc} from hamster brain. **b**, Separated monomer and dimer from the $^{35}S\text{-}PrP^{C}$ used in **a**. 30-40k stands for the 30-40 kDa monomeric PrP and 60k stands for the 60 kDa PrP dimer present as this cell line expresses PrP^{C}. **c**, The same as **a** except that a mutated PrP^{C} with a

stop codon at residue 231 was substituted. This construct lacked the GPI anchor and was expressed in mouse fibroblasts. Molecular weight markers are shown in kilodaltons at the left.

Figure 2.6 Conversion of ^{35}S-PrP^C to the PK-resistant form using PrP^{Sc} pretreated with GdnHCl and GdnSCN. The ^{35}S-PrP^C used here was the GPI^- construct that has been described in Fig. 2.5**c**. Lanes 1-10 contained 40,000 cpm of ^{35}S-PrP^C treated with PK after incubation with 2 μg of PrP^{Sc} pretreated at the indicated condition. Lane 11 contained 10,000 cpm of untreated ^{35}S-PrP^C that was used as substrate in the reaction. Lane 1 is the product of the conversion of ^{35}S-PrP^C with PrP^{Sc} in the absence of GdnHCl. Phosphorimager analysis indicated that the ratio of 35S contained in the PK-resistant conversion products (≥ 17 kDa) in lanes 1-4 was 1:2:6:2, respectively. Molecular weight markers are indicated at the right in kDa.

Figure 2.7 Specificity of the conversion of PrP^C to PK-resistant forms in the presence of PrP^{Sc}. ^{35}S-labeled fibroblast cell lysate proteins "lys" were tested in the conditions used for the conversion of PrP^C. The PrP^C used here was the GPI^- construct mentioned in 2.5**c**. **a**, Combinations of PrP^{Sc}, PrP^c, and "lys" were tested for conversion to a PK-resistant form. "Inc. time" refers to the amount of time the mixture was incubated at 37° C. The ratio of ^{35}S in PrP^C to lysate proteins was approximately 1:15. **b**, Same as **a** but the samples were treated with PK except for lane 1. Equivalent proportions of the reaction mixtures were represented in **a** and **b**, but the fluorographic exposure times for **a** (all lanes), **b** (lanes 1-9) and **b** (lanes 10 and 11) were 2, 12, and 48 hours respectively to best show the bands of interest. To show that PK has worked significantly, lanes **b**1 and **a**2 are the same except **b**1 has been exposed 6 times longer. The asterisk marks the 17 kDa PK-resistant band that resulted from incubation with PrP^{Sc} even in the presence of the lysate proteins. Molecular weights are indicated at the right in kilodaltons.

Figure 2.8 The ^{35}S-PrP product of the conversion reaction was resistant to other proteases and could not be formed using ß-amyloid as a seed. After a two minute incubation, the ^{35}S-PrP^C GPI^- was not resistant to any protease after adding PrP^{Sc} (P) or ß-amyloid (b) (lanes 1-4, top panel). Lanes 5-6 were duplicates of the conversion using PrP^{Sc} and treated with PK. Lane 7 and 8 were PrP^C incubated with ß-amyloid that was pretreated with 0 or 3 M GdnHCl respectively. PK treatment was subsequently performed after the conversion reaction. Lanes 9 and 10 used treatment with chymotrypsin instead of PK after the conversion reaction, but 10 was in the presence of 200 mM $CaCl_2$, which improves the activity of chymotrypsin. Lane 11 has been treated with trypsin instead of PK after the conversion reaction. The bottom panel showed the samples without a protease treatment. Molecular weights are indicated at the left in kilodaltons.

Figure 2.9 Conversion of unglycosylated PrP^{C} to PK-resistant forms. Lanes 1-4 include the construct used in 2.5**a** with or without a treatment of 15 μg/mL tunicamycin during the labeling process. Lanes 3 and 4 were PK treated after a two day incubation with PrP^{Sc}. Lane 5 used the construct used in 2.4**c** and is shown after two day incubation and PK treatment for comparison. The PrP^{C} GPI^{-} construct yielded a prominent band at 17 kDa and the unglycosylated PrP in lane 4 yields a 19 kDa band. The difference is due to the absence of the GPI anchor. Lanes 1 and 2 contained 7 times the counts as shown in the other lanes. Molecular weight markers are shown at the right in kilodaltons.

Fig. 2.1

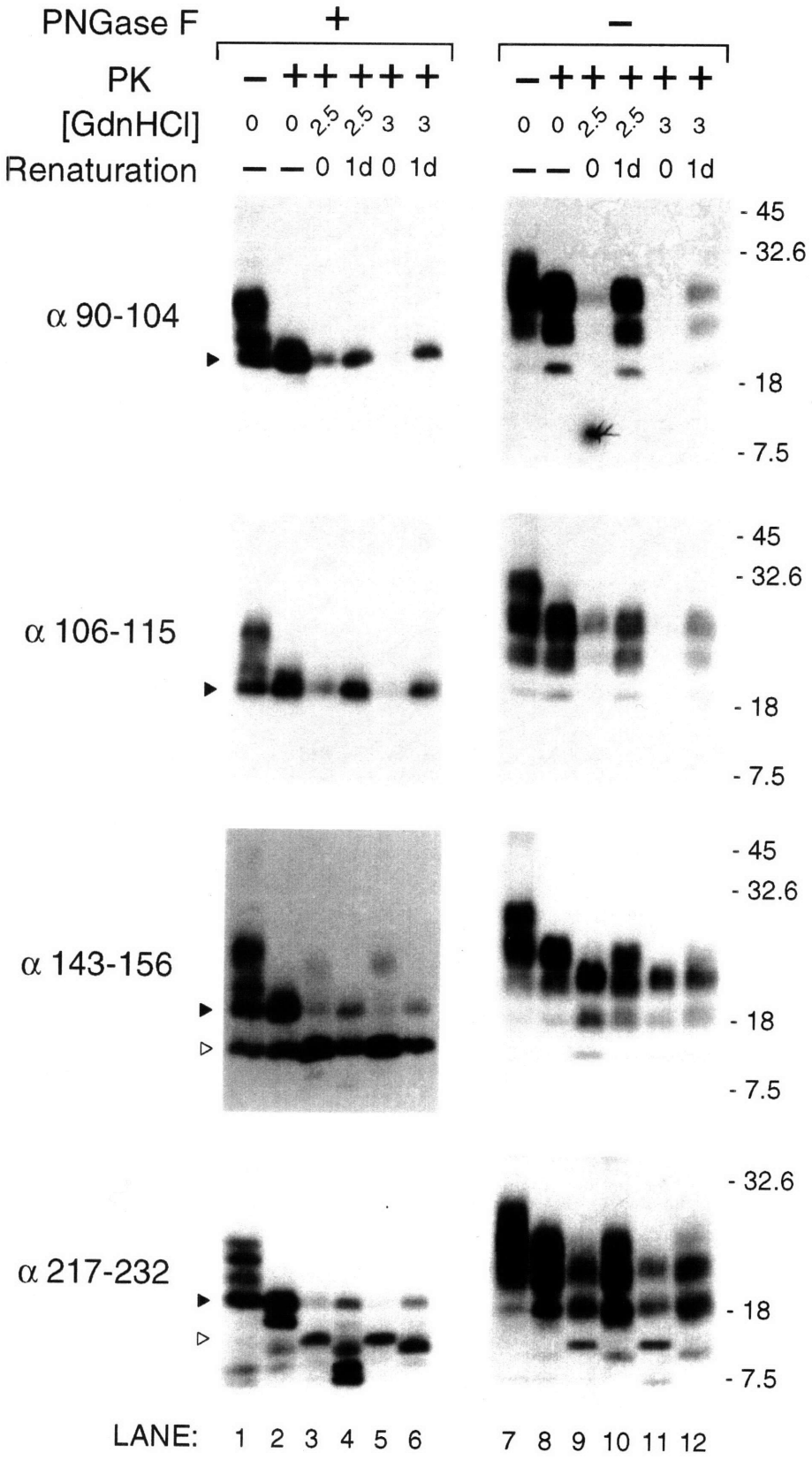

PNGase F
+
−
PK
− + + + + +
− + + + + +
[GdnHCl]
0 0 2.5 2.5 3 3
0 0 2.5 2.5 3 3
Renaturation
− − 0 1d 0 1d
− − 0 1d 0 1d
α 90-104
α 106-115
α 143-156
α 217-232
- 45
- 32.6
- 18
- 7.5
LANE: 1 2 3 4 5 6 7 8 9 10 11 12

Fig.2.2

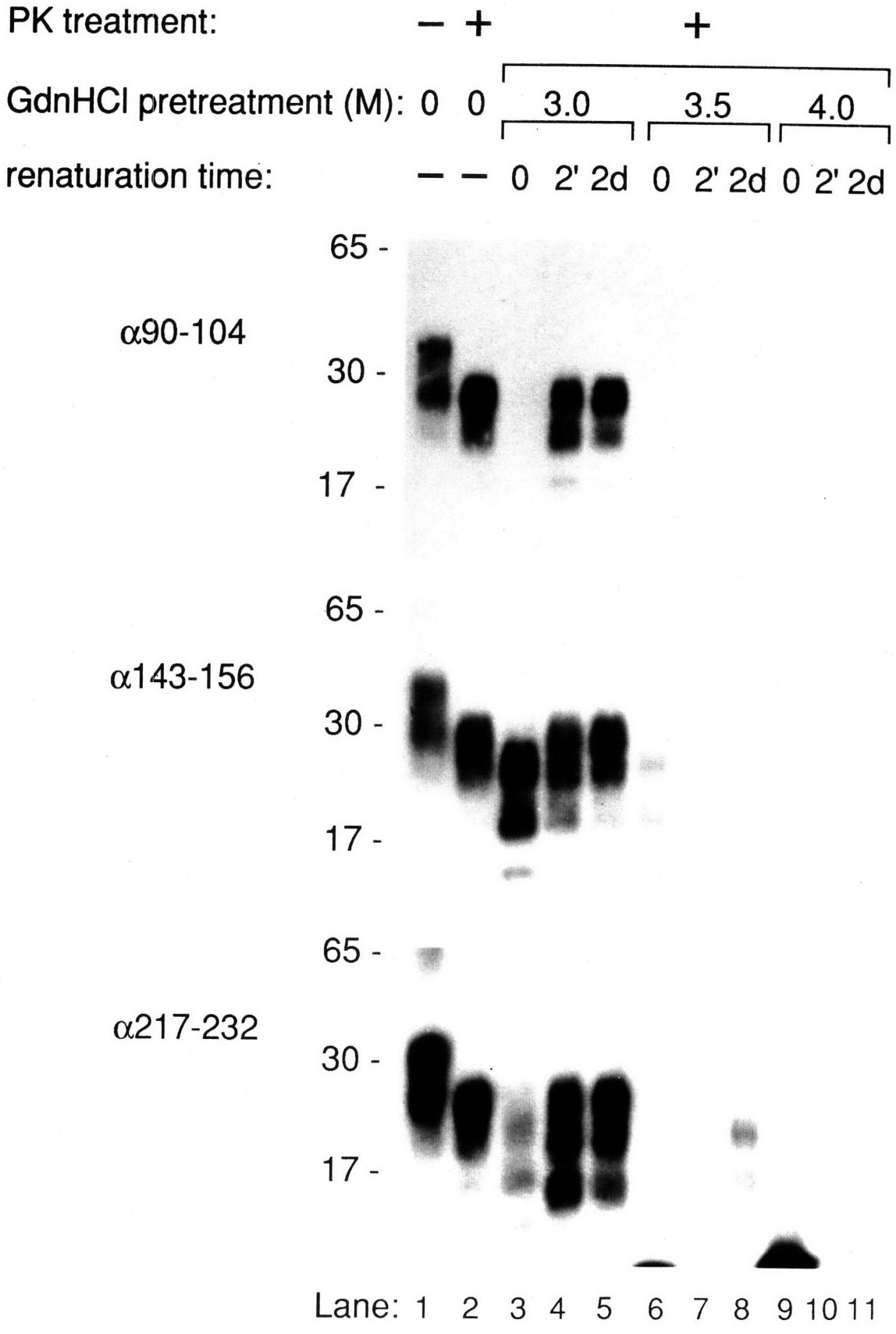
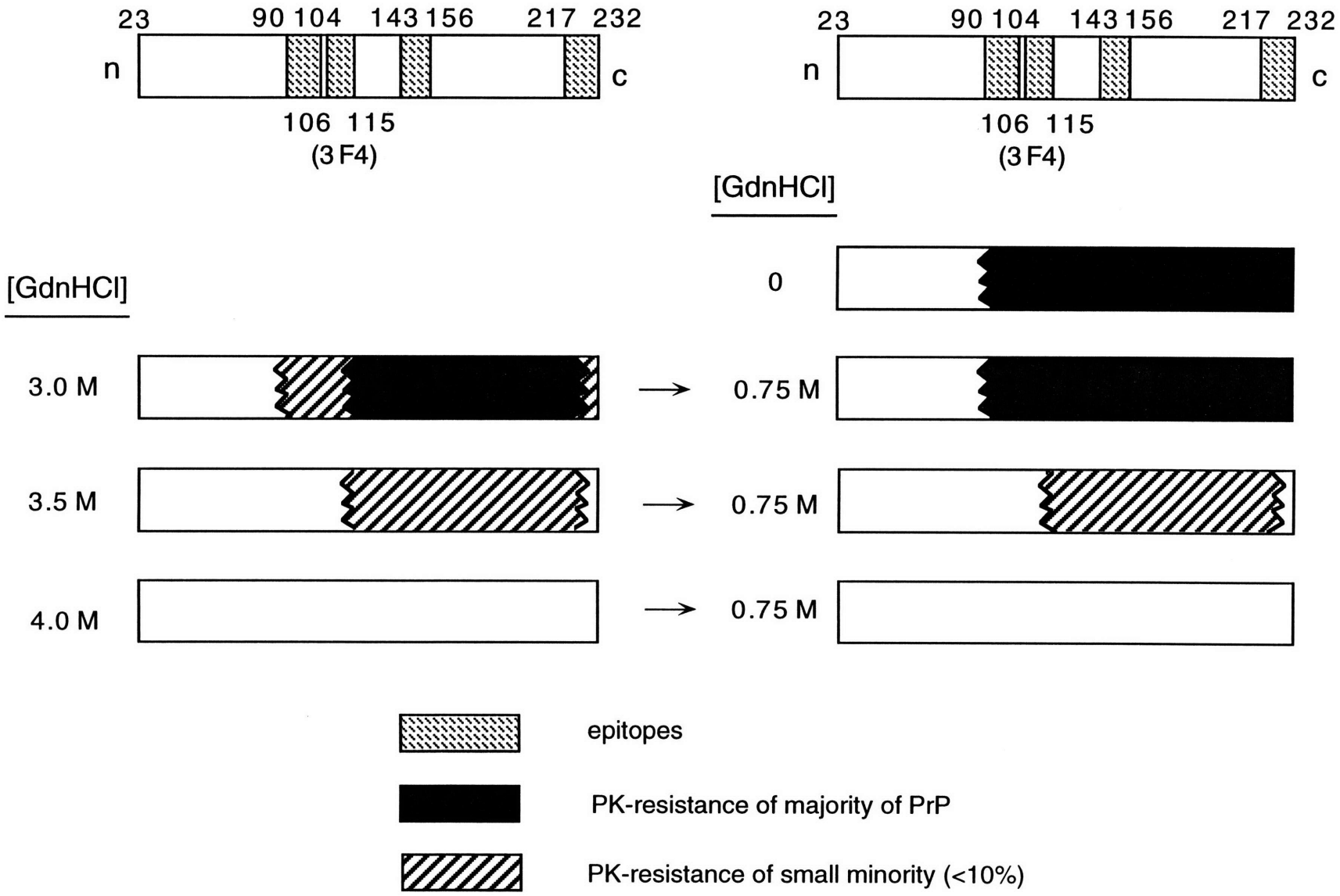

PK treatment: – + +
GdnHCl pretreatment (M): 0 0 3.0 3.5 4.0
renaturation time: – – 0 2' 2d 0 2' 2d 0 2' 2d
65 -
α90-104
30 -
17 -
65 -
α143-156
30 -
17 -
65 -
α217-232
30 -
17 -
Lane: 1 2 3 4 5 6 7 8 9 10 11

Fig. 2.3


23
90 104
143 156
217 232
n
c
106 115
(3F4)
23
90 104
143 156
217 232
n
c
106 115
(3F4)
[GdnHCl]
[GdnHCl]
0
3.0 M
0.75 M
3.5 M
0.75 M
4.0 M
0.75 M
epitopes
PK-resistance of majority of PrP
PK-resistance of small minority (<10%)

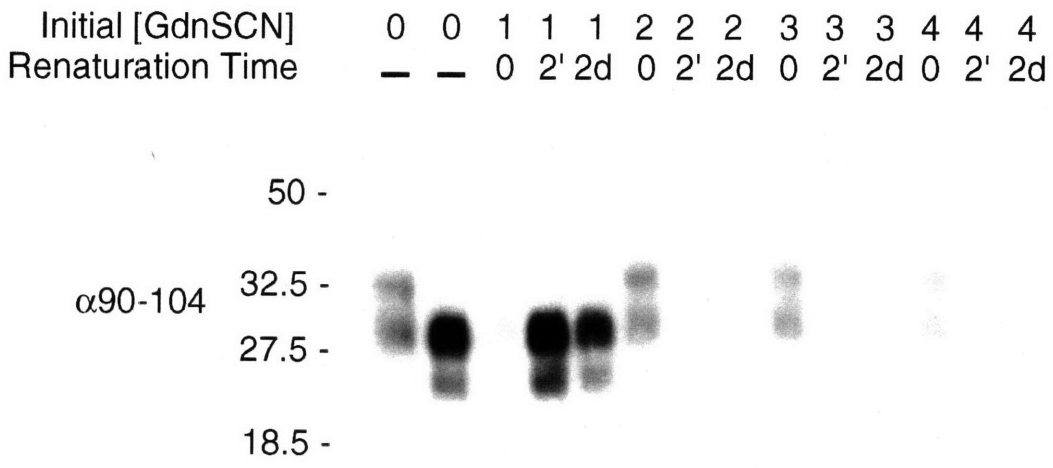
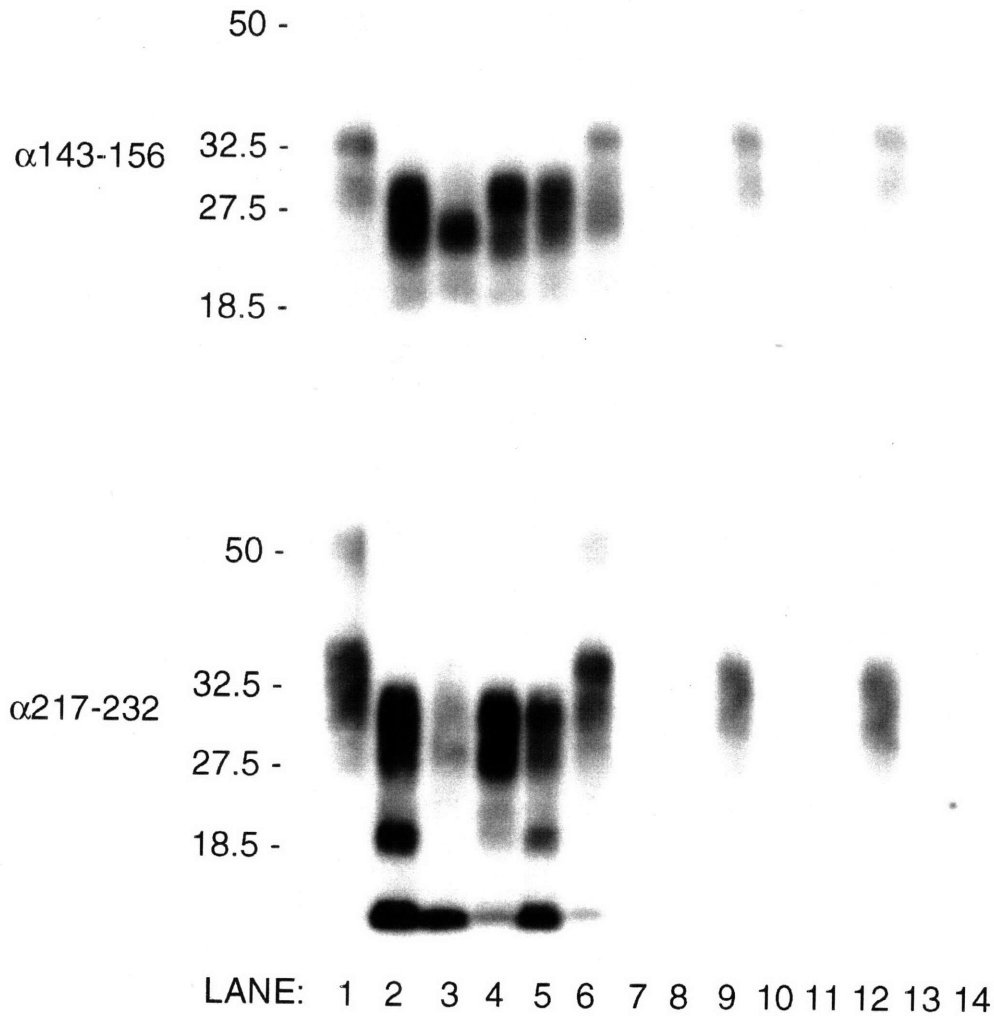

Initial [GdnSCN] 0 0 1 1 1 2 2 2 3 3 3 4 4 4
Renaturation Time — — 0 2' 2d 0 2' 2d 0 2' 2d 0 2' 2d
50 -
α90-104
32.5 -
27.5 -
18.5 -

Fig.2.4


50 -
α143-156
32.5 -
27.5 -
18.5 -
50 -
α217-232
32.5 -
27.5 -
18.5 -
LANE: 1 2 3 4 5 6 7 8 9 10 11 12 13 14

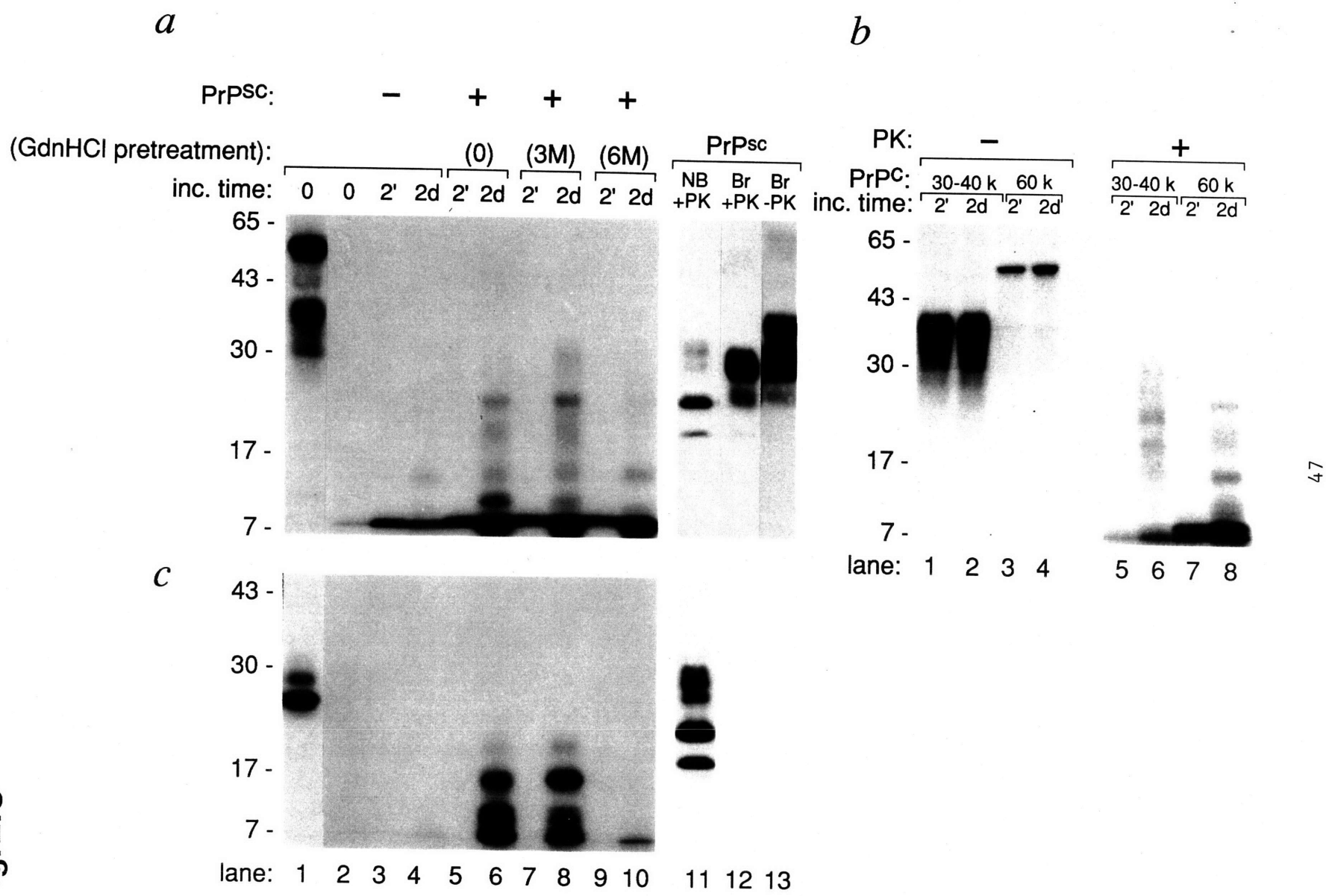

a
b
c
PrPsc:
(GdnHCl pretreatment):
inc. time:
0 0 2' 2d 2' 2d 2' 2d 2' 2d
(0) (3M) (6M)
PrPsc
NB +PK
Br +PK
Br -PK
65 -
43 -
30 -
17 -
7 -
lane: 1 2 3 4 5 6 7 8 9 10 11 12 13
PK:
PrPc:
30-40 k
60 k
2' 2d 2' 2d
lane: 1 2 3 4
30-40 k
60 k
2' 2d 2' 2d
lane: 5 6 7 8

Fig.2.5

Fig. 2.6

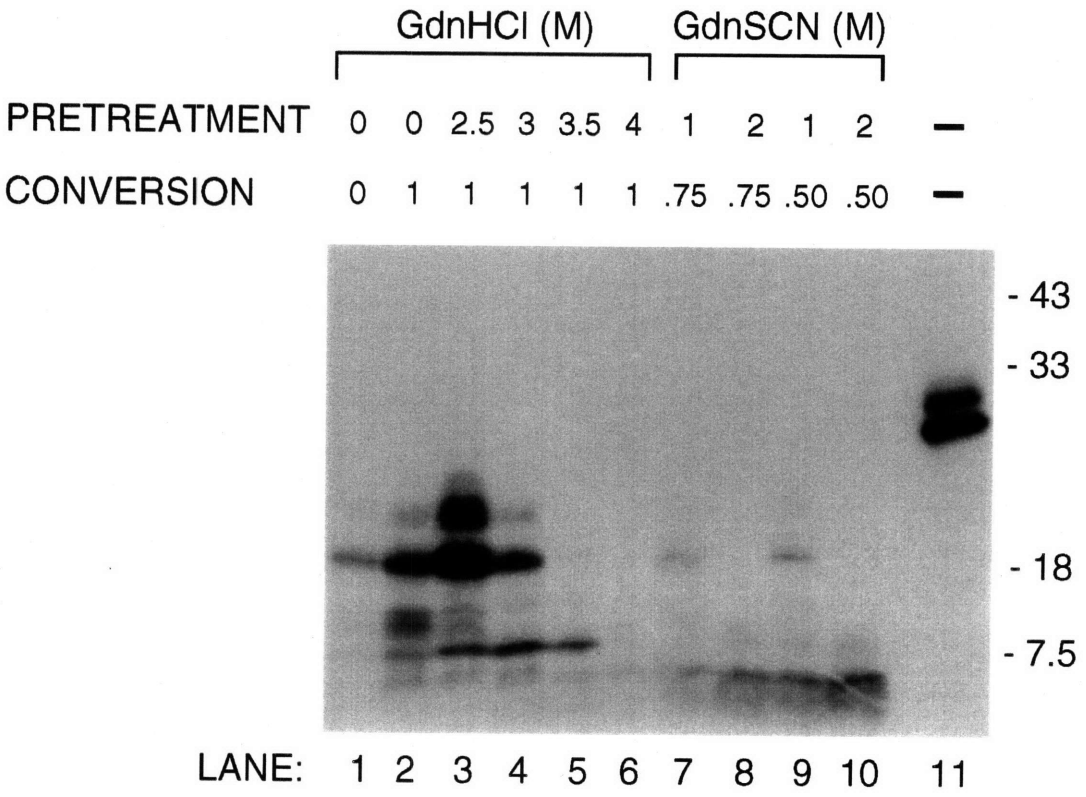

GdnHCl (M)
GdnSCN (M)
PRETREATMENT 0 0 2.5 3 3.5 4 1 2 1 2 —
CONVERSION 0 1 1 1 1 1 .75 .75 .50 .50 —
- 43
- 33
- 18
- 7.5
LANE: 1 2 3 4 5 6 7 8 9 10 11

Fig.2.7

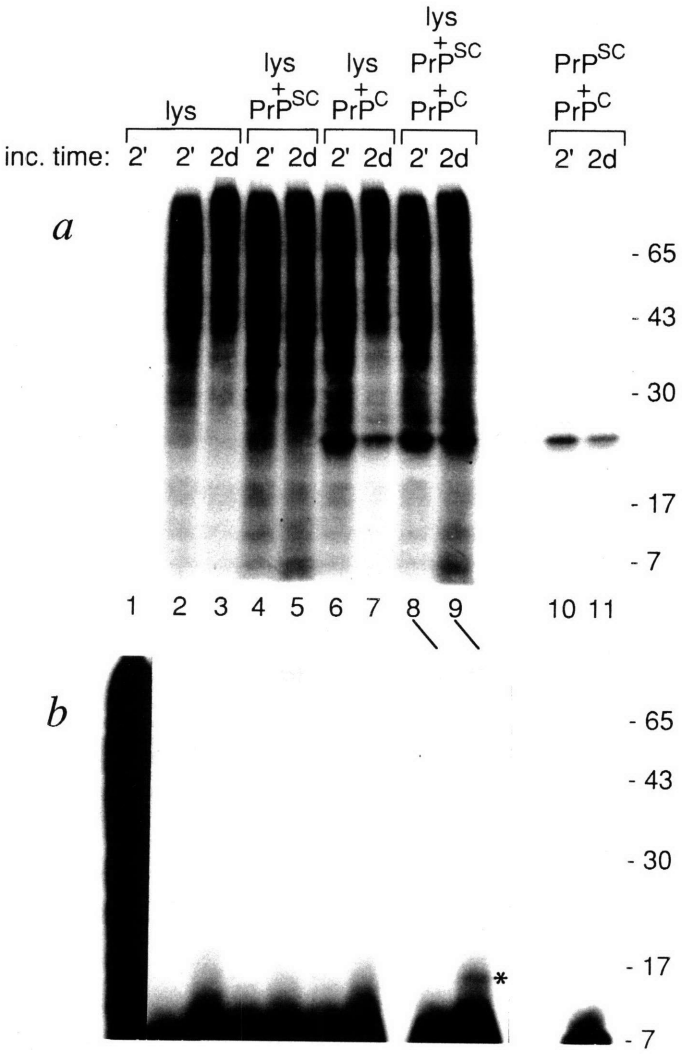

lys
lys + PrP^SC
lys + PrP^C
lys + PrP^SC + PrP^C
PrP^SC + PrP^C
inc. time: 2' 2' 2d 2' 2d 2' 2d 2' 2d 2' 2d
a
- 65
- 43
- 30
- 17
- 7
1 2 3 4 5 6 7 8 9 10 11
b
- 65
- 43
- 30
- 17
- 7
*

Fig.2.8

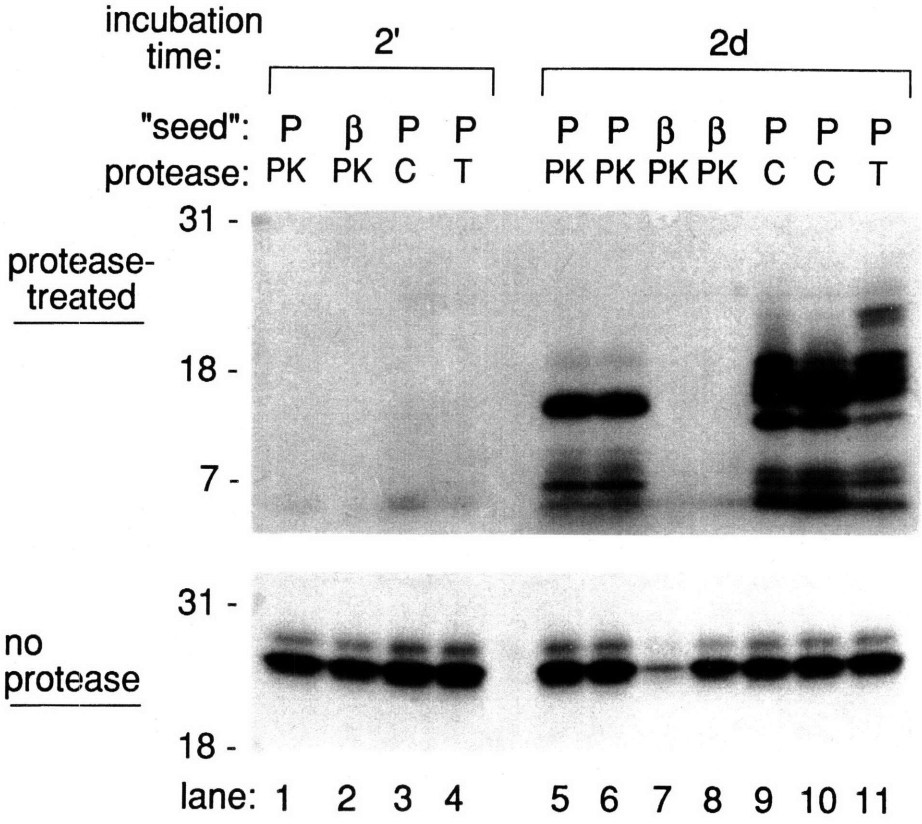
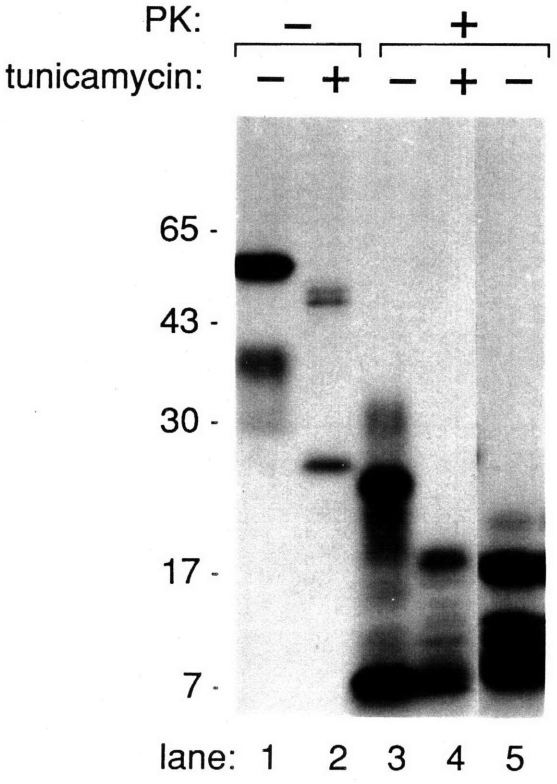

incubation time:
2'
2d
"seed": P β P P P P β β P P P
protease: PK PK C T PK PK PK PK C C T
protease-treated
31 -
18 -
7 -
no protease
31 -
18 -
lane: 1 2 3 4 5 6 7 8 9 10 11

Fig.2.9



Experimental

Purification of PrP^{Sc}

The PrP^{Sc} source was brains of hamsters that had been inoculated with 263K scrapie and were showing signs of the disease. The preparation used was based on the procedure of Bolton et al.[11] Brains from scrapie-infected animals were thawed in phosphate buffered saline (PBS)(10 mM $Na_2HPO_4 \cdot 7H_20$, 10 mM $NaH_2PO_4 \cdot H_2O$, and 130 mM NaCl, pH=6.9) and rinsed three times to eliminate most of the blood. 10% (w/v) sodium N-lauroylsarcosate in TEND (10 mM TrisHCl, 1 mM EDTA, 130 mM NaCl, and 1 mM DTT, pH=8.3) was added to the brains to give a 10% (w/v) solution. The brains were then homogenized and this 10% homogenate solution was put into centrifuge tubes in a Beckman Tl 50.2 rotor and spun at 15K RPM at 4° C for 30 minutes. The supernatant was then transferred to new tubes and spun at 38K RPM at 4° C for 2.5 hours in the 50.2 rotor. The supernatant was discarded and the pellets were combined. The pellets were rinsed three times with 10% NaCl and 1% sulfobetaine 3-14 (SB) in TEND. The combined pellets were then dounce homogenized in 10% NaCl and 1% (w/v) SB in TEND and added to the 50.2 rotor and spun at 45K RPM for 90 minutes at 20° C. Next the supernatant was discarded and the pellet washed with TMCS (10 mM Tris, 5 mM $MgCl_2$, 5 mM $CaCl_2$, and 100 mM NaCl, pH=7.4). The pellet was dounce homogenized in TMCS and DNAse (to 20 μg/mL) and RNAse (to 100 μg/mL) were added. This mixture was gently agitated overnight at 4° C. To this solution was added EDTA to 20 mM, NaCl to 10%, and SB to 1% (w/v). This solution was layered above a cushion of 1 M sucrose, 100 mM NaCl, and 0.5% (w/v) SB and spun at 45K RPM for 90 minutes at 20° C. The supernatant was discarded and the pellet rinsed three times with 1% SB in PBS and then sonicated into 10% NaCl, 1% SB, in TEND in a microcentrifuge tube. This was spun at 75K RPM for 10 minutes at 20° C in a Beckman TLA 100.1 rotor.

The supernatant was discarded and the pellet sonicated into distilled water and centrifuged as just stated. Finally, that supernatant was discarded and the pellet sonicated into 1% SB in PBS. PrP^{Sc} was used directly from the 1% SB in PBS solution.

A 20% acrylamide gel (Pharmacia LKB Phast system) was run and silver stained to determine the purity of a preparation. A BCA protein assay (Pierce) was performed to estimate the total protein content and then immunoblots were performed to confirm the quantity and quality of the PrP^{Sc}

Denaturation of $HaPrP^{Sc}$

To denature $HaPrP^{Sc}$, it was treated so the final concentration was about 1 mg/mL and the denaturant was the desired concentration. The solution was incubated at 37° C for at least 1 hour, but not more than 24 hours. To assess the extent of denaturation (get a time zero point), the PrP^{Sc} solution was diluted with 19 volumes of the same concentration of denaturant, which lowered the PrP^{Sc} concentration to about 50 µg/mL. It was then treated with 50 µg/mL PK directly in the various denaturing conditions for one hour and the sample visualized using SDS-PAGE as stated below.

Renaturation of $HaPrP^{Sc}$ to a $HaPrP^{Sc}$-like form

To renature $HaPrP^{Sc}$ to a $HaPrP^{Sc}$-like form, the denatured PrP solution was diluted with three volumes of TN (10 mM TrisHCl and 100 mM NaCl, pH=7.4 at room temperature) and allowed to incubate at 37° C for between two minutes and two days. To test for the amount of renaturation that occurred, the PrP solution was further diluted with four additional volumes of TN and then treated with PK at a concentration of 50 µg/mL for one hour at 37° C. To inhibit PK, Pefabloc was added to 0.1 mM and the solution let stand for five minutes. SDS-PAGE sample buffer was added (1 mM EDTA, 6% urea (w/v), 5% SDS

(w/v), .05% bromophenol blue (w/v), 4% B-mercaptoethanol (v/v), and 10 mM TrisHCl, pH=8.3) and the samples boiled for five minutes before performing SDS-PAGE. SDS-PAGE was performed using 1 or 1.5 mm 14% acrylamide gels (NOVEX) or 20% acrylamide gels (Pharmacia LKB Phast system). PrP was visualized using an immunoblot procedure. This consisted of several steps: 1. Proteins were transferred to an Immobilon-P transfer membrane (Millipore) using a Milliblot SDE semi-dry transfer system (Millipore). 2. An antibody to a peptide of HaPrP from rabbit serum was added to the membrane and allowed to bind. 3. An anti-rabbit Ig, horseradish peroxidase linked whole antibody (Amersham) was added and allowed to bind with the first antibody. 4. ECL Western blotting reagents (Amersham) were added and the signal was visualized on X-ray film. Note: Distortion of SDS-PAGE may occur if the GdnHCl is not diluted to <30 mM in the SDS-PAGE sample.

PNGase F treatment of denatured and renatured PrP^{Sc}

PrP^{Sc} was treated with PNGase F (New England Biolabs) after the final methanol precipitation, after all other manipulations of the denaturing and renaturing treatment were finished. The PrP^{Sc} methanol precipitate was taken up in 20 µL of 88% formic acid and after 10 minutes on ice, rotary evaporated to dryness. 20 µL of water, SDS (to 0.5% w/v), and 2-mercaptoethanol (to 1% w/v) were added and the sample was boiled for 5 minutes. NP-40 to 1% (v/v) and 10 µL of sodium phosphate buffer were added. 2 µL of the supplied enzyme solution were added and incubated at 37° C overnight. Sample buffer was added to the solution and SDS-PAGE performed as before.

^{35}S-Labeling and Purification of PrP^{C}

The PrP^{C} converted in the cell-free reaction was ^{35}S-labeled and immunoprecipitated in the following manner. A 25 cm^2 flask of two-thirds to

three-fourths confluent cells was used and first washed 3 times with PBBS. For one hour at 37° C the cells were preincubated with 1.5 mL Met/Cys deficient growth medium. Note: methionine and cysteine are each at 0.1X normal concentration in RPMI medium. 1 mCi ^{35}S-Expre^{35}S^{35}S label (NEN #Neg-072) was added and incubated for 90 minutes at 37° C. The cells were then rinsed 3 times with cold PBBS and then lysed. The lysing buffer used was 1 mL of 0.5% (w/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 5 mM TrisHCl pH 7.4 at 4° C, 150 mM NaCl, and 5 mM EDTA. Cell nuclei and debris was spun out by centrifuging at 1000*g* for 5 minutes at 4° C. 4 volumes of methanol (-20° C) were then added to the supernatant and left at -20° C for an hour to precipitate. A 15 minute spin at 2500*g* was used to remove the precipitate. The precipitate was sonicated into 1 mL DLPC buffer as described in Borchelt et al.[12] 3 µL of 3F4 ascites was added and left at 4° C overnight. 30 µL of 10% (w/v) protein A sepharose beads were added and gently agitated at 4° C for one hour. The protein A sepharose beads were then washed one time with 1 mL DLPC, twice with TN-1% N-lauroylsarcosine, and finally once with water. Residual liquid was drawn off with a Hamilton narrow bore syringe. PrP was eluted with 25 µL of 3-7.5 M GdnHCl. The beads were then gently agitated for 10 minutes at 37° C and the eluate drawn off. This elution step was repeated. The first elution typically had more counts per minute than the second but often it contained enough to be useful. The eluted protein was used directly in the conversion reaction. The labeled PrP was scintillation counted to determine the amount of labeling. To purify tunicamycin treated PrP, 15 µg/mL tunicamycin was added to the cells as the ^{35}S was added and then immunoprecipitated in the same way.

Conversion of ^{35}S-PrP^{C} to the protease-resistant form

To convert the ^{35}S-labeled PrP^{C} to the protease-resistant form, PrP^{Sc} was denatured in 2.5-3 M GdnHCl and then mixed with the labeled PrP^{C}. A solution

of 2 μg/μL PrP^{Sc} was mixed with an equal volume of 5-6 M GdnHCl and then incubated at 37° C for as little as 1 hour, but not more than 24 hours. Equal volumes of denatured PrP^{Sc} and the labeled PrP^{C} solutions were mixed and then diluted to 0.75 M GdnHCl using TN (130 mM NaCl, 50 mM TrisHCl, pH=7.4 at room temperature). Mixing was done so that PrP^{Sc} was never in more than the desired pretreatment GdnHCl concentration. The solution was then incubated at 37° C for at least 16 hours.

The PK-resistance of the labeled PrP was then tested after further dilution with TN by treating with 50 μg/mL of PK for one hour at 37° C. Trypsin and chymotrypsin at 50 μg/mL for one hour were also substituted for PK. PK was inhibited using Pefabloc. 20 μg of thyroglobulin as a carrier protein was added and the samples were precipitated in four volumes of methanol at -20° C. The resulting pellet was boiled in sample buffer and run on SDS-PAGE (14% acrylamide gel). The ^{35}S was visualized by soaking the gel in Entensify autoradiography enhancer (DuPont), drying the gel, and exposing to X-ray film.

A typical reaction was:

2 μL of 1 mg/mL $HaPrP^{Sc}$ in 3 M GdnHCl
2 μL $HaPrP^{C}$ with 30,000 cpm/μL in 3 M GdnHCl
12 μL TN

This was briefly sonicated and incubated. It was then diluted with 64 μL TN and 4 μL of 1 mg/mL PK was added. After an hour of incubation at 37° C, 20 μL of 5X Pefabloc, 4 μL of 5 mg/mL thyroglobulin, and 4 volumes of methanol were added. After an hour at -20° C, the tube was spun for 15 minutes at 15K RPM in a Beckman microcentrifuge. The pellet was sonicated and boiled into 20 μL of the 1X urea sample buffer used above and run on SDS-PAGE.

Isolation of PrP^{C} monomer and dimer from D4 cells

To isolate monomer, a 25 cm^2 flask of D4 cells was first labeled as previously mentioned. The cells were washed three times in PBBS and 1 mL of

PBBS was added along with 9 µL of phosphatidylinositol-specific phospholipase C (PIPLC) (ICN) that was [0.1 u/µL]. The cells were then incubated for 20 minutes with occasional rocking. The supernatant was removed and spun at 1000 RPM for 5 minutes in a JS 4.2 Beckman rotor. The supernatant was then immunoprecipitated directly with 3F4 as previously mentioned.

To isolate the PrP^C dimer, D4 cells were rinsed three times in cold PBBS. They were lysed with 1 mL of lysing buffer as stated above. Cell debris was removed by spinning at 1500 RPM for 5 minutes in a JS 4.2 Beckman rotor. The supernatant was then layered over a 5% sucrose pad and spun at 90K RPM for 45 minutes in a TL100.3 Beckman rotor. The pellet was then sonicated into 1 mL of DLPC and immunoprecipitated using 3F4 as mentioned above.

Conversion to the protease-resistant form in the presence of lysate proteins

The conditions used were identical to those mentioned above except for the addition of labeled proteins that remained in the cell lysate after immunoprecipitation. In this instance the lysate proteins were treated in 3 M GdnHCl before mixing with PrP^{Sc} and labeled PrP^C and then dilution, incubation, etc. The amount added was based on the number of counts in the lysate. Typically, 60,000 cpm of ^{35}S in PrP^C and 1,000,000 cpm in lysate proteins were added.

REFERENCES FOR CHAPTER 2

1. Safar, J., Roller, P.P., Gajdusek, D.C. & Gibbs, C.J. *J. Biol. Chem.* **268**, 20276-20284 (1993).
2. Caughey, B., Race, R.E., Ernst, D., Buchmeier, M.J. & Chesebro, B. *J. Virol.* **63**, 175-181 (1989).
3. Taraboulos, A., Rogers, M., Borchelt, D.R., McKinley, M.P., Scott, M., Serban, D. & Prusiner, S.B. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 8262-8266 (1990).
4. Rogers, M., Yehiely, F., Scott, M. & Prusiner, S.B. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 3182-3186 (1993).
5. Weissmann, C. *Nature* **352**, 679-683 (1991).
6. Caughey, B. & Raymond, G.J. *J. Virol.* **67**, 643-650 (1993).
7. Kocisko, D.A., Come, J.H., Priola, S.A., Chesebro, B., Raymond, G.J., Lansbury, P.T. & Caughey, B. *Nature* **370**, 471-474 (1994).
8. Raeber, A.J., Borchelt, D.R., Scott, M. & Prusiner, S.B. *J. Virol.* **66**, 6155-6163 (1992).
9. Prusiner, S.B., Groth, D., Serban, A., Stahl, N. & Gabizon, R. *Proc. Natl. Acad. Sci. USA* **90**, 2793-2797 (1993).
10. Kaneko, K., Peretz, D., Pan, K.-M., Blochberger, T.C., Willie, H., Gabizon, R., Griffith, O.H., Cohen, F.E., Baldwin, M.A. & Prusiner, S.B. *Proc. Natl. Acad. Sci. USA* **92**, 11160-11164 (1995).
11. Bolton, D.C., Bendheim, P.E., Marmorstein, A.D. & Potempska, A. *Arch. of Biochem. Biophysics* **258**, 579-590 (1987).
12. Borchelt, D.R., Taraboulos, A. & Prusiner, S.B. *J. Biol. Chem.* **267**, 16188-16199 (1992).

Chapter 3
Aggregates of PrP^{Sc} induce the cell-free conversion of PrP^{C} to the protease-resistant state

The inability to separate PrP^{Sc} and scrapie infectivity has made PrP^{Sc} the prime candidate as the infectious agent.[1-3] Supporting this idea are observations from chapter 2 that PrP^{C} will convert to the PK-resistant form when incubated with preexisting PrP^{Sc} in a cell-free system. As mentioned in the last chapter, it is not known yet whether new scrapie infectivity is generated upon conversion of PrP^{C} to these PK-resistant forms. The cell-free system was next used to probe the mechanism of the conversion of PrP^{C} to PrP^{Sc}. If PrP^{Sc} is all or part of the infectious agent then this conversion is central to the infection process.

Any mechanistic model for PrP^{Sc} formation must address two questions regarding this conversion reaction. First, what catalyzes the PrP^{C}-to-PrP^{Sc} conversion (the "converting activity")? According to the heterodimer/unfolding model, the catalyst (PrP^{Sc}) is a monomeric conformational isomer of PrP that catalyzes the conformational change *via* a PrP^{C}-PrP^{Sc} heterodimer.[3-5] Alternatively, the seeded polymerization or nucleation model holds that the catalyst is an ordered multimeric aggregate of PrP (PrP^{Sc}) which acts as a nucleus for polymerization of PrP.[6, 7] Second, what is the rate determining step in the PrP^{C}-to-PrP^{Sc} conversion? The heterodimer/unfolding model holds that the rate determining step is a conformational change. In the nucleation model the rate determining step is the formation of a multimeric seed of PrP^{Sc} which directs further PrP polymerization. TSE infection would then be the transfer of preformed PrP^{Sc} nuclei which instigate conversion in the new host. Attempts to

determine the mechanism of PrP^{Sc} formation are complicated by the fact that the reaction itself and the physical state of PrP^{Sc} is difficult to monitor *in vivo*. However, the *in vitro* system described above allows more direct characterization of the species capable of inducing the conversion of PrP^{C} to PrP^{Sc}-like protease-resistant forms. This chapter describes experiments assessing the concentration dependence of the reactants in the conversion reaction and the size of the molecular species associated with converting activity by sedimentation and ultrafiltration analysis.

Concentration-dependence of the cell-free conversion of ^{35}S-PrP^{C} to the protease resistant form.

The extent of conversion of ^{35}S-PrP^{C} to protease-resistant, PrP^{Sc}-like species was followed as a function of time and the amount of PrP^{Sc} added to the reaction mixture (Figs. 3.1**a**,**b**). In this reaction, ^{35}S-PrP^{C} immunoprecipitated from uninfected fibroblast cells was mixed with scrapie brain-derived PrP^{Sc} in 2.5 M GdnHCl and then diluted to 0.75 M GdnHCl to initiate the conversion reaction. After the incubation period, the products of the reaction were treated with PK to test for the conversion of ^{35}S-PrP^{C} to PK-resistant forms. For quantitative comparisons, "conversion" was defined as the formation of 17-21-kDa PK-resistant ^{35}S-PrP since these were of the size expected of PK-digested PrP^{Sc} derived from this type of PrP^{C} precursor, i.e., 6-7 kDa lower in molecular weight than the full-length 24-28 kDa ^{35}S-PrP^{C} precursor (e.g., see Fig. 3.3**a**). Fig. 3.1**b** illustrates the increase in conversion as either the initial PrP^{Sc} or incubation period were increased. The extent of conversion at 22 hours was determined over a wider range of PrP^{Sc} concentrations (Fig. 3.2**c**). Increasing PrP^{Sc} concentration always increased the extent of conversion although at high PrP^{Sc} concentration (> *ca.* 100μg/mL), the concentration-dependence was less

marked. At PrP^{Sc} concentrations from 5-62 μg/mL, a plot of % conversion versus initial PrP^{Sc} concentration was essentially linear. Extrapolation of such a plot to % conversion =0 resulted in a non-zero x-intercept (*ca.* 5.1 μg/mL, 150 nM), providing evidence that a threshold concentration of PrP^{Sc} was required for the existence of converting activity. The threshold concentration was also supported by the fact that conversions attempted with PrP^{Sc} concentrations less than 5 μg/mL gave no detectable product (data not shown). The value of the apparent threshold concentration reflects the fact that the *in vitro* conversion reaction was conducted in the presence of GdnHCl and, thus, may be different (presumably lower) in the absence of GdnHCl. The dependence of the total conversion at 22 hours on ^{35}S-PrP^{C} concentration was also determined (Fig. 3.1**e**). At a constant PrP^{Sc} concentration (125 μg/mL), the formation of ^{35}S-labeled conversion product was directly dependent on the initial ^{35}S-PrP^{C} concentration. Extrapolation to 0% conversion demonstrated no threshold concentration. It is important to note that, due to technical obstacles, the ^{35}S-PrP^{C} concentration range examined was *ca.* 1% of the PrP^{Sc} concentration range tested in Fig. 3.1**b** (see Experimental section).

Sedimentation properties of the converting activity

In chapter 2 it was stated that pretreatment of PrP^{Sc} with 2.5-3M GdnHCl enhances the converting activity. Such GdnHCl treatments are also known to disaggregate PrP^{Sc}.[8, 9] To determine if converting activity was associated with an effectively solubilized PrP^{Sc} fraction[1] or a residual PrP^{Sc} aggregate, the sedimentation properties of the converting activity was analyzed in the presence

[1]Treatments with GdnHCl can alter the biochemical properties (i.e. PK-resistance and tendency to aggregate) of PrP^{Sc} that are usually used to define PrP^{Sc} and distinguish it from PrP^{C}. Nonetheless, to define the origin of samples derived from PrP^{Sc} preparations, they will continue to be refered to as PrP^{Sc} with the acknowledgement that the PrP in these samples might no longer have the defining properties of PrP^{Sc}.

of GdnHCl. PrP^{Sc} pretreated with 3 M GdnHCl was centrifuged under conditions predicted to pellet 90% of particles of $S_{20,w}$ = 12 (based on calculations described in Experimental section). The pellet and supernatant fractions were tested for converting activity (Fig. 3.2**a**) and analyzed for PrP content (without PK treatment) by immunoblotting (Fig. 3.2**b**). The vast excess of PrP^{Sc} over PrP^{C} in the reaction dictates that the PrP detected by immunoblot was derived predominantly from PrP^{Sc}. A majority of the PrP was found in the supernatant, but converting activity was found only in the pellet. This indicated that, although the 3 M GdnHCl treatment effectively solubilized more than half of the original PrP^{Sc} preparation and increased the overall converting activity, only sedimentable aggregates had converting activity.

To further compare the sedimentation rates of converting activity and GdnHCl-solubilized PrP^{Sc}, a series of sedimentation velocity centrifugations was performed (Fig. 3.3). In this experiment, PrP^{Sc} pretreated in 2.5 M GdnHCl was used because preliminary experiments with this particular PrP^{Sc} preparation indicated that the converting activity was optimal with this pretreatment. The sample was placed over a layer of 5% sucrose in 2.5 M GdnHCl and centrifuged at various speeds. Fractions of the gradient were tested for converting activity (Fig. 3.3**a,d**) and analyzed by immunoblotting for total PrP content (Fig. 3.3**b**). With spins of 2000 and 8000 x g, a majority of the converting activity remained in the top (T) fraction corresponding to the sample zone (Fig. 3.3**a,d**). A spin at 70,000 x g cleared >95% of converting activity from the sample zone (T). Calculations based on the clearing factor (k) for this rotor speed and sample zone radius estimated that 90% of particles of $S_{20,w}$ = 17 would be cleared from the sample zone under these conditions. A 234,000 x g spin cleared all detectable converting activity from both the sample zone and middle (M) fractions. At this speed, $\geq$ 90% of particles of $S_{20,w} \geq 5$ would be cleared from the sample zone. In

contrast to the converting activity, at least half of the PrP detected by immunoblot remained in the top (sample) zone after these spins (Fig. 3.3**b**). This confirmed that the converting activity was associated with sedimentable aggregates rather than with the nonsedimenting PrP^{Sc} generated by the GdnHCl treatment.

A set of single-subunit molecular weight standard proteins [lactalbumin (14 kDa), trypsin inhibitor (20 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), and phosphorylase b (94 kDa); data not shown] and thyroglobulin (669 kDa, with 335 kDa subunits; Fig. 3.3**c**) were centrifuged at 70,000 and 234,000 x g in identical gradients containing 2.5 M GdnHCl, but none sedimented through the gradient to the extent observed for the converting activity at those centrifugal forces. Native thyroglobulin is 19S, but the effect of 2.5 M GdnHCl on the sedimentation properties of thyroglobulin and the other protein molecular weight standards is not known. The sedimentation behavior of blue dextran (2,000 kDa) was closest to that of the converting activity, but the clearance of the blue dextran absorbance from the sample (T) zone still was slower that of the converting activity [Figures 3.3**a**,**c** & **d**] Thus, the detectable converting activity sedimented faster than the solubilized PrP (monomeric MW = 25 - 30 kDa), other proteins up to 669 kDa (or 335 kDa if thyroglobulin subunits are dissociated in 2.5 M GdnHCl) and 2,000-kDa blue dextran.

To address the possibility that solubilization of converting activity might be achieved at a different GdnHCl concentration, PrP^{Sc} was treated in 0, 1.5, 2.0, 2.5 and 3.0 M GdnHCl and centrifuged at 234,000 x g in gradients formed with a matching concentration of GdnHCl. In all cases, the detectable converting activity was cleared completely from the sample zone and sedimented to the bottom (B) and pellet (P) fractions (data not shown), indicating that the

converting activity was associated with aggregates regardless of the GdnHCl concentration. GdnHCl concentrations greater than 3 M were not tested because, as noted in chapter 2, converting activity is unstable at concentrations $\geq$ 3.5 M and not renaturable by subsequent dilution of the GdnHCl.

Sedimentation of PK-treated converting activity and PrP^{Sc}

Treatment of the PrP^{Sc} with PK results in the loss of approximately half of the total PrP and removal of 67 N-terminal amino acids (6-7 kDa) from the remaining PrP^{Sc} to generate a predominantly 27-30 kDa protein (PrP 27-30) (Fig. 3.4).[10, 11] In addition, PK treatment degrades much of the large molecular weight (>40 kDa) material that is observed by SDS-PAGE with typical PrP^{Sc} preparations (PrP 27-30 is > 90% pure as determined by SDS-PAGE). Since PK treatment does not eliminate the converting activity associated with PrP^{Sc} (see chapter 5), PK pretreatment was tested to see whether it would allow the converting activity to be more readily disaggregated by GdnHCl. PrP 27-30 was treated with 2.5 M GdnHCl and subjected to a 234,000 x g gradient centrifugation identical to that performed with the untreated PrP^{Sc} preparation. Analysis of the converting activity in the gradient fractions indicated that it pelleted (Fig. 3.5**a**) as it did prior to PK digestion (Figs. 3.2 & 3.3). Analysis of the total PrP by immunoblot indicated that, in contrast to the situation with the untreated PrP^{Sc}, none of the PrP 27-30 was effectively solubilized by the GdnHCl (Fig. 3.5**b**). This result suggested that PK digestion of PrP^{Sc} removed the PrP species that could be effectively solubilized away from the PrP^{Sc} aggregates associated with converting activity by 2.5 M GdnHCl.

Ultrafiltration of converting activity in GdnHCl

As another test of the size of molecular species associated with converting activity, ultrafiltration was performed on PrP^{Sc} (not treated with PK) in 3 M GdnHCl. PrP^{Sc}-derived PrP was retained by a filter with a nominal molecular weight limit (NMWL) rating of 100 kDa (not shown) but passed through a 300 kDa NMWL filter (Fig. 3.6**b**). In contrast, the converting activity was retained by the 300 kDa NMWL filter (Fig. 3.6**a**). Thus, the 300 kDa NMWL filter separated the converting activity from the bulk of the solubilized PrP^{Sc}, providing additional evidence that the converting activity is associated only with aggregates larger than the dissociated PrP. Since the maximum pore size of the 300 kDa NMWL filter is estimated by the manufacturer to be 30 nm, the Stokes radius of the inactive PrP that passed through this filter must be < 30 nm. The Stokes radius of the converting activity must be larger than that of the PrP^{Sc} without converting activity; however, one cannot definitively conclude that it is greater than 30 nm because of possible polarization layer effects and the likelihood that the average pore size of the filter is much lower than 30 nm.

Similar filtration experiments performed with filters with nominal maximum pore sizes of 100-450 nm indicated that converting activity could pass through the filters (data not shown). The passage of converting activity through the 100 nm (maximum pore size) filter indicated that it can have a Stokes radius of $\leq$ 100 nm.

Ultrafiltration of PK-treated converting activity in GdnHCl

Ultrafiltration of PK-treated PrP^{Sc} in 3 M GdnHCl indicated that the converting activity was retained by both 100- and 300-kDa NMWL filters as was the case with the untreated PrP^{Sc} (Fig. 3.7**a**). In contrast to the untreated PrP^{Sc}, little (<15%) of the PK-treated PrP^{Sc} passed through the 300 kDa NMWL filter (Fig. 3.7**b**). This was consistent with the sedimentation analysis of PK-treated

PrP^{Sc} (Fig. 3.5**b**), which also indicated reduced solubilization by GdnHCl compared to the untreated PrP^{Sc}.

PK-resistance and renaturation of PrP^{Sc} are associated with GdnHCl-insoluble aggregates

Having detected converting activity only in the more aggregated PrP^{Sc} fractions generated by filtration or centrifugation in 2.5-3 M GdnHCl, these same fractions were tested for resistance to PK in GdnHCl. They were also tested for the ability to recover PK resistance after dilution of the GdnHCl. This was done very similarly to the procedure mentioned in chapter 2 using different antibodies to PrP epitopes. In Fig. 3.8 samples of unfractioned, supernatant, and pelleted PrP^{Sc} were immunoblotted without PK treatment to compare the amount of PrP present. A 234,000 x g, 30 minute spin that should clear particles of S>5 divided the PrP approximately in half (compare lanes 2 and 3). Samples of supernatant material were treated with PK in the presence of 3 M GdnHCl and no PrP was detected by any of the antibodies (lane 4). The protease resistance of the pelleted material in the presence of 3 M GdnHCl was similar to the unfractioned material (lane 6). Supernatant and pelleted fractions were then diluted to 0.75 M GdnHCl and allowed to incubate overnight before treatment with PK. Again there was no PK-resistant PrP visualized by any antibody from the supernatant fraction (lane 5). However, most of the PrP^{Sc} in the pellet recovered its PK-resistance indicating that renaturation of this aggregated PrP^{Sc} had occurred (lane 7). These results suggest that PrP^{Sc} must be aggregated to have protease resistance.

Association of converting activity with PrP^{Sc} aggregates

The cell-free reaction for converting PrP^{C} to PK-resistant forms has made it possible to begin characterizing the components and mechanism of the

converting activity under defined conditions. The dependence of conversion rate on the amount of PrP^{Sc} in the reaction mixture establishes that a component of the PrP^{Sc} preparation is involved in the rate-determining step. The sedimentation and filtration of the converting activity indicated that it was associated with aggregates that range in size from being at least several times larger than solubilized PrP to macroscopic particles. The sedimentation and filtration data provided complementary information about the sizes of species with converting activity. The sedimentation rate of a particle is a function of both its size and shape. Thus, one might argue that subtle differences in sedimentation of PrP species could be explained by differences in conformation rather than aggregation state and that converting activity is associated with a more compact, rapidly sedimenting conformer. However, if this were the case, the more compact conformer would also be expected to pass more readily through the pores of an ultrafiltration membrane than a more extended conformer with a larger Stoke's radius. The opposite result was observed, which is consistent only with the converting activity being associated with an aggregate of larger mass than the solubilized PrP. This conclusion was confirmed by the observation that the detectable converting activity sediments faster than other molecules up to 50 times greater in molecular weight than monomeric PrP.

The nature and origin of the PrP without converting activity or protease-resistance that was solubilized from the original PrP^{Sc} aggregate is not clear, but GdnHCl-soluble subfractions of PrP^{Sc} have also been observed by other investigators.[8, 9] Since these PrP molecules appear to be more sensitive to PK than the residual PrP 27-30 aggregate, they may be PrP^{C} molecules that are bound (normally or artifactually) to PrP^{Sc} without being fully integrated and converted to PK-resistant state characterized by PrP 27-30. The stimulation of converting activity by the GdnHCl treatments of PrP^{Sc} may be due to the

removal of these nonconverted PrP molecules which may be "abortive complexes" that block access to active catalytic surfaces for the conversion reaction. The presence of these GdnHCl soluble PrP molecules might also contribute substantially to the PrP^C precursor concentration once the GdnHCl is diluted for the conversion reaction. However, this solubilization would have the effect of greatly diluting the specific radioactivity of the PrP^C, which would likely diminish, rather than stimulate, the observed formation of PK-resistant ^{35}S-PrP conversion products. Another possibility for the increase in converting activity is that GdnHCl solubilizes some cofactor that is needed for the conversion.

Ramifications regarding models of PrP^{Sc} formation

Evidence on the sizes of species that contain converting activity is useful in discriminating between various theories for the mechanism of PrP^{Sc} formation. The observation that a wide range of PrP aggregates possess converting activity is consistent with the nucleation model. As in the case of amyloid formation, any aggregate larger than the critical nucleus would be a competent seed for polymerization.[7] The nucleus, which is defined as the least stable intermediate along the aggregation pathway, must be an ordered oligomer.

The observation of a threshold concentration for converting activity is also consistent with the nucleation proposal, in which the converting activity is a seed for the ordered aggregation of PrP. The threshold concentration may correspond to the critical concentration which is observed for protein polymerization processes known to occur *via* a nucleation-dependent mechanism (e.g., microtubule formation, flagellum formation, sickle cell fibril formation).[12-15] Below the critical concentration, the predominant species is the monomer. The

nucleus is, by definition, the highest energy species along the polymerization pathway and the smallest possible seed for polymerization.[7] Since the nucleus is almost completely dissociated below the critical concentration, one would not expect to see conversion. However, above the critical concentration, the predominant species are large aggregates, in equilibrium with the monomer. All of these post-nucleus aggregates are, in theory, capable of seeding the polymerization. The non first-order concentration dependence seen at high PrP^{Sc} concentration may be due to ultrastructural effects, for example, fibril bundling, which may occur in those concentration ranges. Bundling could decrease the efficiency of the seed by blocking the active growth faces.[7] The threshold concentration observed (*ca.* 150 nM) may reflect the solubility of PrP^{Sc} under the conditions of the *in vitro* conversion reaction (0.75 M GdnHCl). The solubility of PrP^{Sc} under more physiological conditions would probably be lower.

In contrast, the present data are not consistent with the heterodimer model, which postulates that the active species is a monomer. Although PrP^{Sc} is usually isolated in rapidly sedimenting aggregates, the aggregation of PrP^{Sc} may be largely an artifact of purification and that the active species is a PrP^{Sc} monomer (or small oligomer) that dissociates as a discrete unit from the aggregate.[3] The cell-free conversion assay has given us the opportunity to directly test for converting activity in subfractions of PrP^{Sc} preparations treated with a wide range of conditions (from 0-3 M GdnHCl) with varying tendencies to solubilize PrP from the preexisting PrP^{Sc} aggregate. In all cases, converting activity was detected only in the aggregate fractions rather than in supernatants or filtrates containing dissociated PrP^{Sc}. These data are inconsistent with the idea that the active PrP^{Sc} species is solely a monomer or small oligomer. Finally, the threshold concentration effect would not be expected of a monomeric species.

It is instructive to consider separately a heterotrimer model, which postulates that the converting activity is a PrP^{Sc} dimer. The concentration-dependence of dimer formation would be second-order, not first-order as observed. In addition, a dissociating dimer could pass through the filter in monomeric form and reassociate in the filtrate. A nondissociating dimer, analogous to the dimeric form of CD2 which has recently been characterized,[16] would show a first-order concentration dependence, but not a threshold concentration. A dimer with an extremely high association equilibrium constant could produce a curve which may resemble the one seen in Fig. 3.1**d**, however, it is clear from the sedimentation experiments that a PrP^{Sc} dimer is not the major converting species. Nonetheless, these results cannot absolutely rule out the possibility that small oligomers, such as dimers or trimers have some limited (undetected) converting activity.

If PrP^{Sc} were active as a monomer, then this sizing data would require that it be attached to a non-PrP molecule(s) or aggregate of much greater size to account for its sedimentation and filtration properties. Although the complete composition of these aggregates is not clear, silver stain and immunoblotting analyses of fractions containing converting activity indicate that PrP^{Sc} is the predominant protein component, especially after PK digestion (Fig. 3.4).[17] Therefore, the particulate behavior of the converting activity is likely due to an association with PrP^{Sc} aggregates rather than with PrP^{Sc} monomers bound to a much larger mass of other molecules. These results do not rule out the possibility that some non-PrP molecules have gone undetected or could be minor constituents of the aggregates. This is especially plausible because of the vast excess of PrP^{Sc} over $^{35}S\text{-}PrP^{C}$ in the present conversion reaction. For instance, glycosaminoglycans and other possible ligands (e.g. pentraxins, chaperonins, apolipoprotein E, NAC, nucleic acids) have been postulated to play roles in the

formation or stabilization of PrP^{Sc}.[18-21] These factors might be present and active in the PrP^{Sc} preparations at substoichiometric levels as part of the conversion mechanism. It can be concluded from the present study that any such factor must co-purify with GdnHCl-resistant PrP^{Sc} aggregates and be resistant to PK.

Reconciling the properties of the in vitro converting activity with those of the scrapie infectious agent

Given the hypothesis that the activity that induces PrP^{Sc} formation is the infectious agent of scrapie, it is relevant to consider how the aggregated nature of the *in vitro* converting activity compares to previous analyses of the size of the scrapie agent. Many studies have indicated that scrapie and Creutzfeldt-Jakob disease infectivity is associated with large sedimentable (>40 S) particles. However, other analyses have indicated that much smaller sized particles can be infectious.[22-24] For instance, scrapie associated fibrils (SAF) or prion rods are not visible by electron microscopy in all infectious preparations (e.g. liposome preparations).[22, 25] However, it is possible that the low concentrations of small PrP aggregates required to nucleate polymerization might escape detection, especially in liposomes. Furthermore, the increased infectivity of a liposome-PrP^{Sc} suspension relative to a sample containing the same amount of PrP in the form of amyloid fibrils[25] is consistent with the nucleation model in which smaller PrP aggregates would have a higher converting activity per PrP molecule. It must be emphasized that the nucleated polymerization model requires only that the seed be ordered, not that it have the morphological properties of an amyloid fibril. The sensitivity of the scrapie agent to inactivation by ionizing radiation is consistent with the agent being a PrP dimer of *ca.* 55 kDa.[23] However, it is also consistent the agent being a substantially larger nucleus. Many *oligomeric* enzymes have produced monomeric target sizes, indicating that energy transfer

between subunits need not occur and that the activity of each subunit can be independent of the activity of its neighbors in the oligomer.[26-28] Accordingly, if the scrapie agent is an aggregate of PrP, the interpretation of the radiation target size data will be complicated by the fact that a multimeric PrP seed may be resistant to radiation damage of subunits that are not part of the growth face. The observed target size of 55 kDa may reflect a growth face defined by two PrP molecules within the context of a larger polymer.

Figure 3.1 The PrP^{Sc}- and time-dependence of the conversion reaction. **a.** Converting activity. PK-resistant ^{35}S-PrP species generated from the incubation of ^{35}S-PrP^{C} with different concentrations (μg/mL) of PrP^{Sc} for various lengths of time (hr) as described for the conversion reaction in Experimental. The PrP^{C} used in this study lacks a GPI anchor and is predominantly unglycosylated (24 kDa), with a minority monoglycosylated (28 kDa) (see Fig. 3.3**a**, for example). The bracket on the left and in subsequent figures indicates the PrP^{Sc} -like 17-21 kDa bands that were used in the quantitative comparisons described below. All lanes represent reactions initiated with 60,000 cpm of ^{35}S-PrP^{C}. Molecular weight markers are indicated at the right in kDa. **b.** Percent of ^{35}S-PrP^{C} converted to 17-21 kDa PK-resistant species (mean ± S.D. of triplicate determinations) after incubation with designated PrP^{Sc} concentrations as a function of incubation time (includes data shown in **a**). Only bracketed bands as shown in A were counted as converted product using the Phosphorimager. **c.** Percent conversion in 22 hr versus [PrP^{Sc}]. **d.** Expanded plot of linear region of data in panel **c** showing a line fit by simple linear regression with an X-intercept of 5.1 μg/mL (0.15 μM PrP^{Sc}, based on a molecular weight of 35 kDa). **e.** Plot of the total 16-20 kDa conversion product versus initial [^{35}S-PrP^{C}]. Semi-quantitative immunoblotting was used to estimate the total PrP^{C} as 10 ng/60,000 CPM. The [PrP^{Sc}] and incubation time were constant in these reactions at 125 μg/mL and 22 hours respectively. The percentage of the initial ^{35}S-PrP^{C} that was converted was approximately 20% over the entire range tested.

Figure 3.2 Comparison of the converting activity in aggregated versus solubilized PrP^{Sc}. **a.** Converting activity (as described in legend to Fig. 3.1**a**) in aliquots of unseparated (total) PrP^{Sc} in 3 M GdnHCl or of pellet or supernatant fractions of the same after centrifugation at 217,000 x g max. **b.** Immunoblot showing relative total PrP content of same fractions used in the conversion reaction in **a**. A polyclonal rabbit antiserum raised against a synthetic peptide

corresponding to hamster PrP residues 90-104 was used. Molecular weight markers are indicated at the right in kDa.

Figure 3.3 Sedimentation analysis of GdnHCl treated converting activity and PrP^{Sc}. **a**. Converting activity (as described in legend to Fig. 3.1**a**) in aliquots of unfractionated (total) PrP^{Sc} in 2.5 M GdnHCl or top (T), middle (M), bottom (B), and pellet (P) fractions (as indicated by diagram at left) after centrifugation at the designated g force. One third of the ^{35}S-PrP^{C} used in each conversion reaction is shown without PK digestion in the PrP^{C} lane. Molecular weight markers are indicated at the right in kDa. **b**. Immunoblot of the total PrP in fractions described in **a**. **c**. Sedimentation of thyroglobulin (669 kDa total, 335 kDa subunits) or blue dextran (2000 kDa) in 2.5 M GdnHCl on identical gradients. **d**. Clearance of the converting activity from the sample (T) zone after centrifugation at the designated relative centrifugal forces (RCF). The ordinate is the percent reduction in the 16-20 kDa PK-resistant ^{35}S-labeled, PK-resistant PrP species generated in the conversion reaction by aliquots of the T fractions as compared to the total unfractionated PrP^{Sc} sample. The data points show the mean $\pm$ range of measurements from two independent experiments (including the one in panel a), except in the case of the 2,000 x g spin which is from a single determination. For comparison, the calculated $S_{20,w}$ values of particles that would be 90% cleared from the sample zone under the conditions of each centrifugation are shown.

Figure 3.4 SDS-PAGE with silver staining of PrP^{Sc} before and after treatment with PK as described in Experimental. The thin band seen just above 30 kDa in the third lane was likely PK itself. The 1/5 dilution refers to a 5 fold decrease in the protein concentration loaded onto the gel after PK treatment. Molecular weight markers are indicated at the left in kDa.

Figure 3.5 Sedimentation of converting activity and PrP^{Sc} after PK treatment. PrP^{Sc} was treated with PK as described in Experimental section, incubated at 1 mg/mL for 5 hr at 37° C in 3 M GdnHCl and fractionated at 234,000 x g max on a gradient identical to those described in Fig. 3.3. **a**. Converting activity (as described in the legend to Fig. 3.1**a**) in unfractionated (total) K-treated PrP^{Sc} in 2.5 M GdnHCl or top (T), middle (M), bottom (B), and pellet (P) fractions. One third of the ^{35}S-PrP^{C} used in each conversion reaction is shown without PK digestion in the PrP^{C} lane. **b**. Immunoblot of the total PrP in fractions described in **a**. Molecular weight markers are indicated at the right in kDa.

Figure 3.6 Ultrafiltration of PrP^{Sc} in 3 M GdnHCl and measurement of converting activity. Filtrate and retentate fractions were obtained from a 300 kDa NMWL filter as described in Experimental section. **a**. Converting activity in equivalent aliquots of the retentate, filtrate and unfractionated sample (total). **b**. Immunoblot of the total PrP in same fractions. Molecular weight markers are indicated at the right in kDa.

Figure 3.7 Ultrafiltration of PK-treated PrP^{Sc} in 3 M GdnHCl and assessment of converting activity. The PK-treated PrP^{Sc} described in Fig. 3.5 was incubated in 3 M GdnHCl at a concentration of 1 mg/mL and filtered as described in Fig. 3.6 except that 100 kDa NMWL filters were also used. **a.** Converting activity in equivalent aliquots of the indicated fractions. One third of the ^{35}S-PrP^{C} used in each conversion reaction is shown without PK digestion in the PrP^{C} lane. **b.** Immunoblot of the total PrP in same fractions. Molecular weight markers are indicated at the right in kDa.

Figure 3.8 PK-resistance and refolding of soluble vs. aggregated fractions of PrP^{Sc}. PrP^{Sc} in 3 M GdnHCl was fractionated by ultracentrifugation at 234,000 x g as described in Experimental. Fractions are designated as whole or unfractioned (w), supernatant (s), or pelleted (p). The first three lanes are to compare the total amounts of PrP in each fraction without PK-treatment. The supernatant and pellet fractions were treated with PK in 3 M GdnHCl (renaturation time = 0) or diluted to 0.75 M and incubated for one day before further dilution and treatment with PK as described in Experimental.

Fig.3.1A

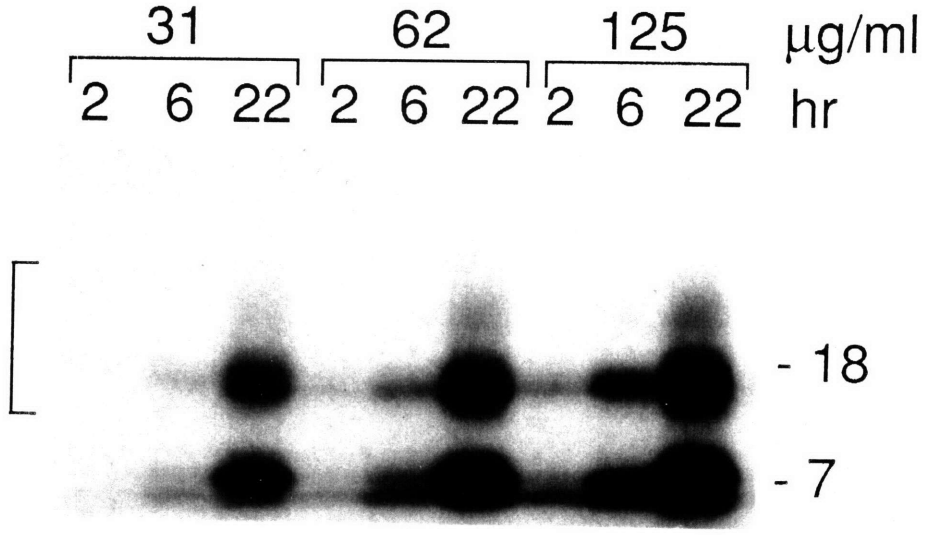


Fig. 3.1 B

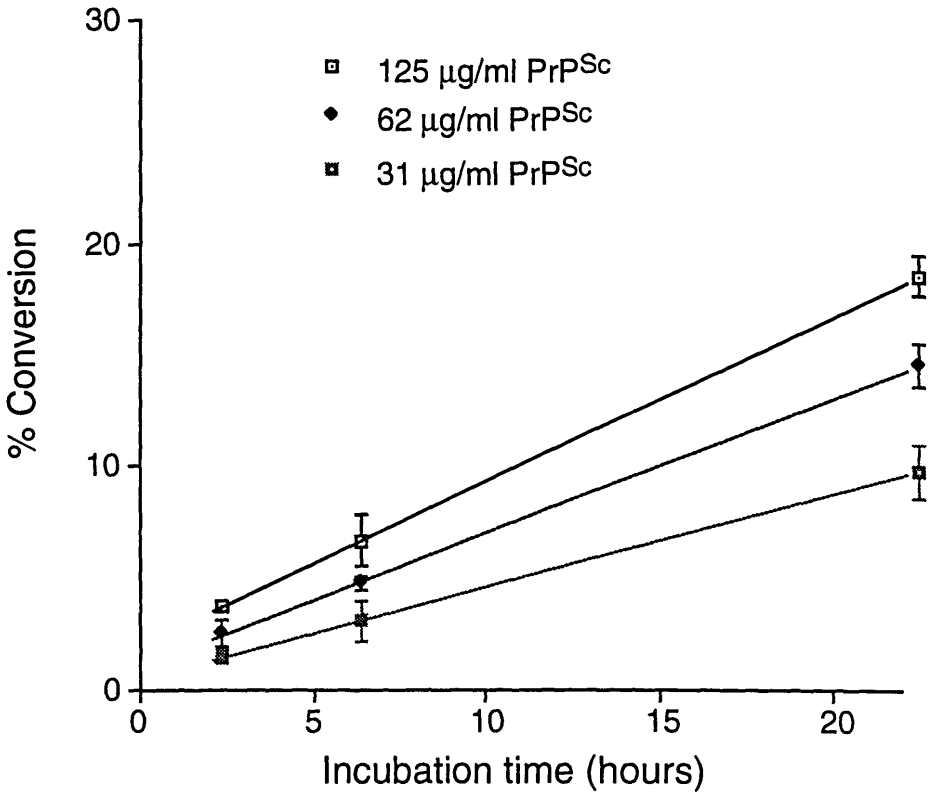

125 µg/ml PrP^Sc
62 µg/ml PrP^Sc
31 µg/ml PrP^Sc
% Conversion
Incubation time (hours)
0
10
20
30
0
5
10
15
20

Fig.3.1C

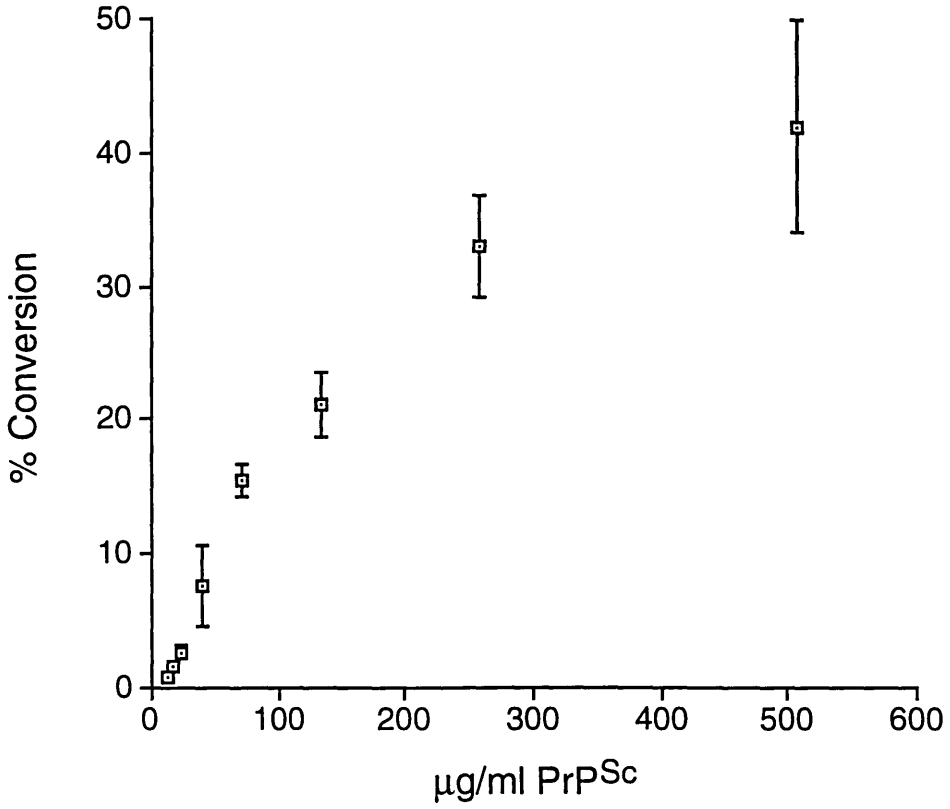


Fig.3.1 D

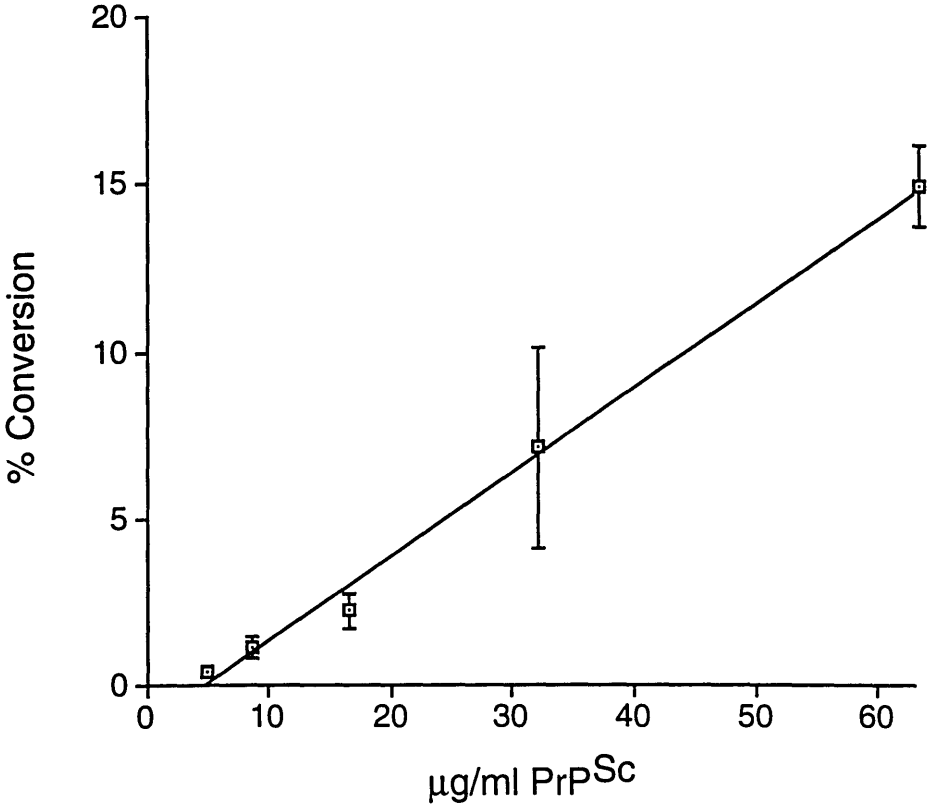


Fig.3.1E

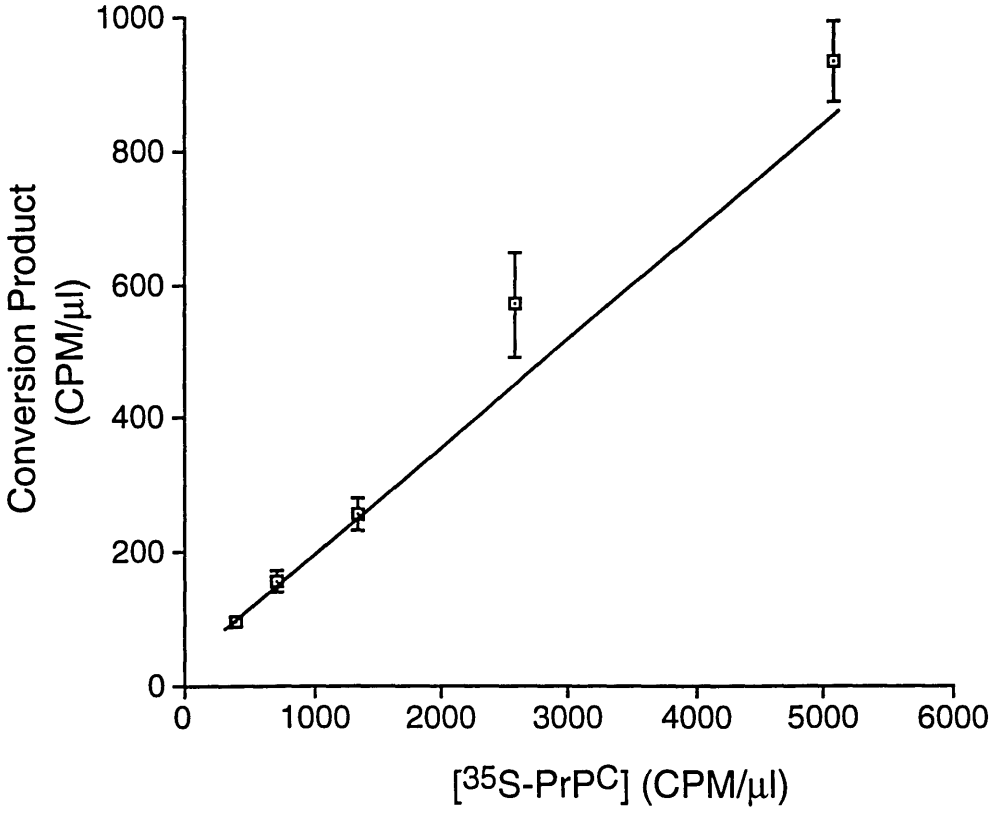

1000
800
600
400
200
0
Conversion Product
(CPM/µl)
0
1000
2000
3000
4000
5000
6000
[35S-PrPC] (CPM/µl)

Fig.3.2

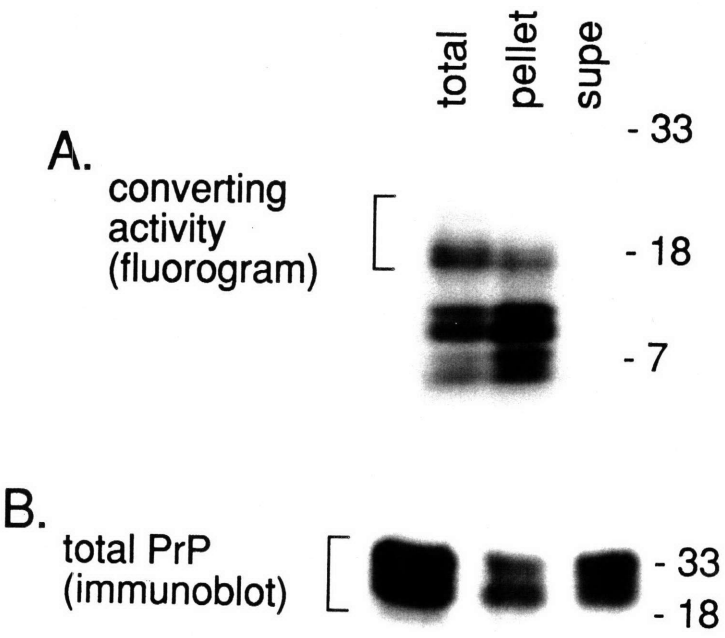

total
pellet
supe
A.
converting
activity
(fluorogram)
- 33
- 18
- 7
B.
total PrP
(immunoblot)
- 33
- 18

Fig. 3.3

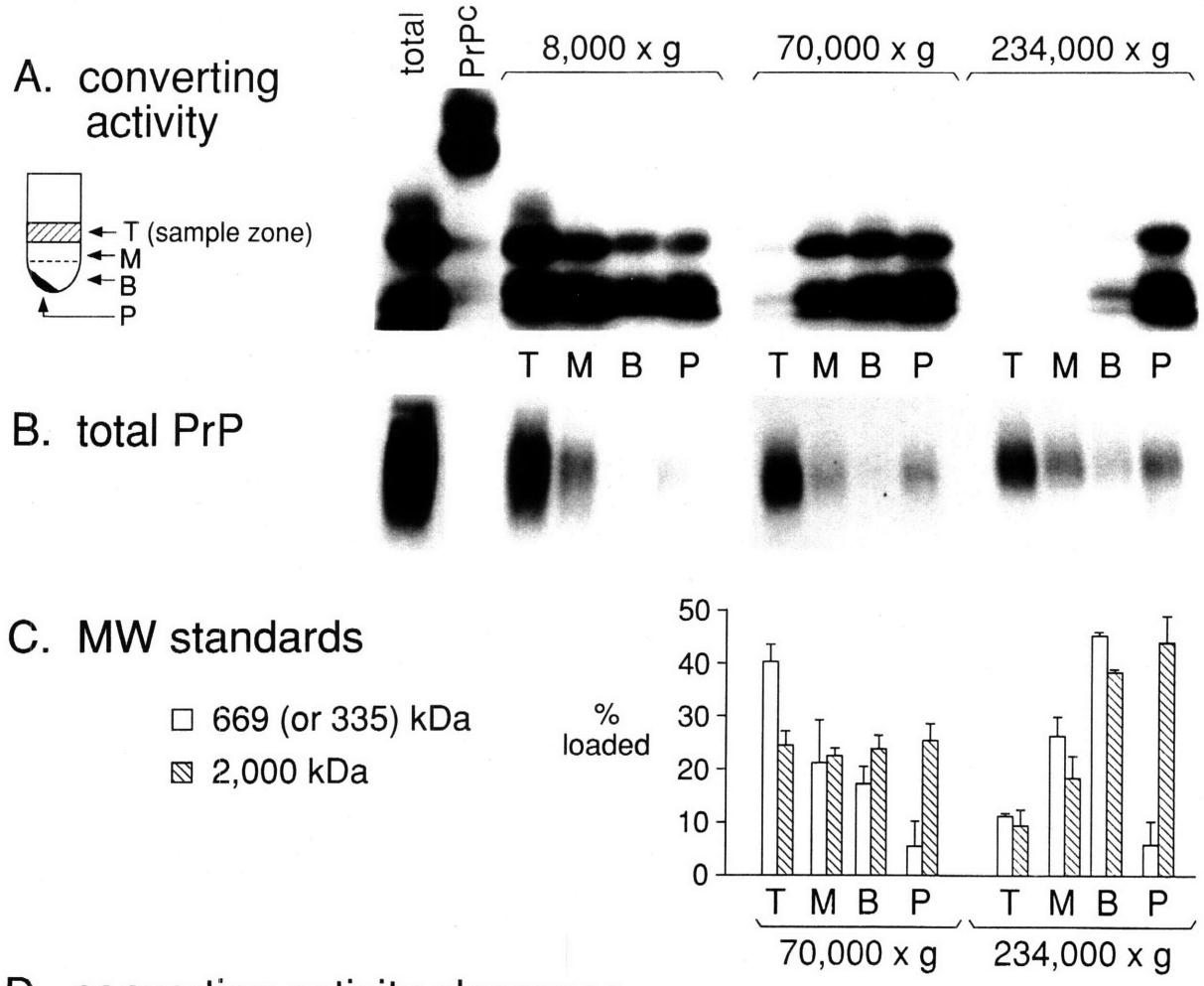

A. converting activity
T (sample zone)
M
B
P
total
PrPc
8,000 x g
70,000 x g
234,000 x g
T M B P T M B P T M B P
B. total PrP
C. MW standards
669 (or 335) kDa
2,000 kDa
% loaded
50
40
30
20
10
0
T M B P T M B P
70,000 x g
234,000 x g
D. converting activity clearance
% cleared from sample zone(T)
100
80
60
40
20
0
2
8
70
234
1000 x g RCF
>610
≥153
≥17
≥5
estimated $S_{20,W}$

Fig. 3.4

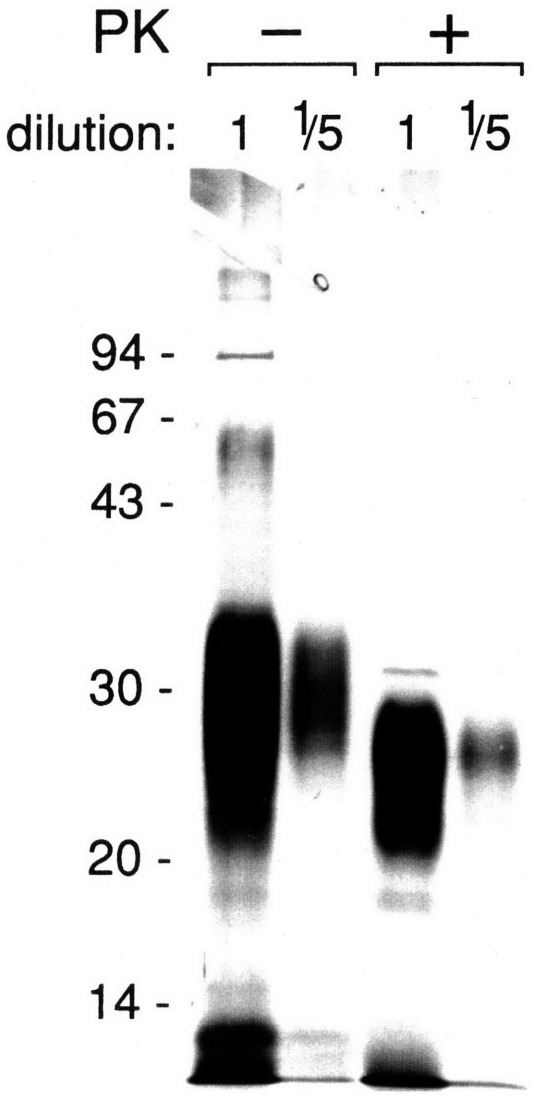


Fig. 3.5

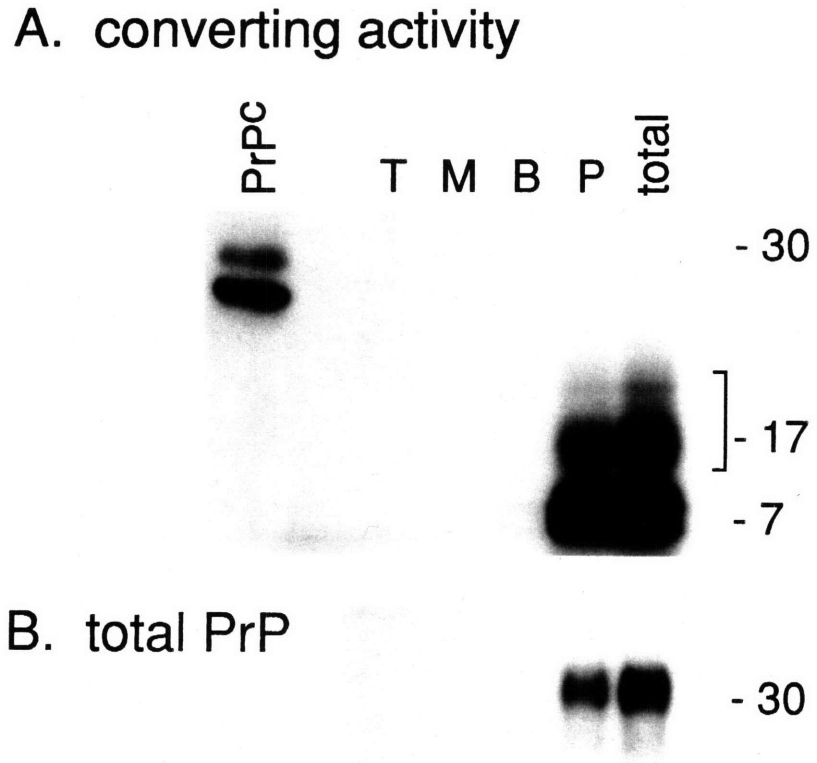


Fig.3.6

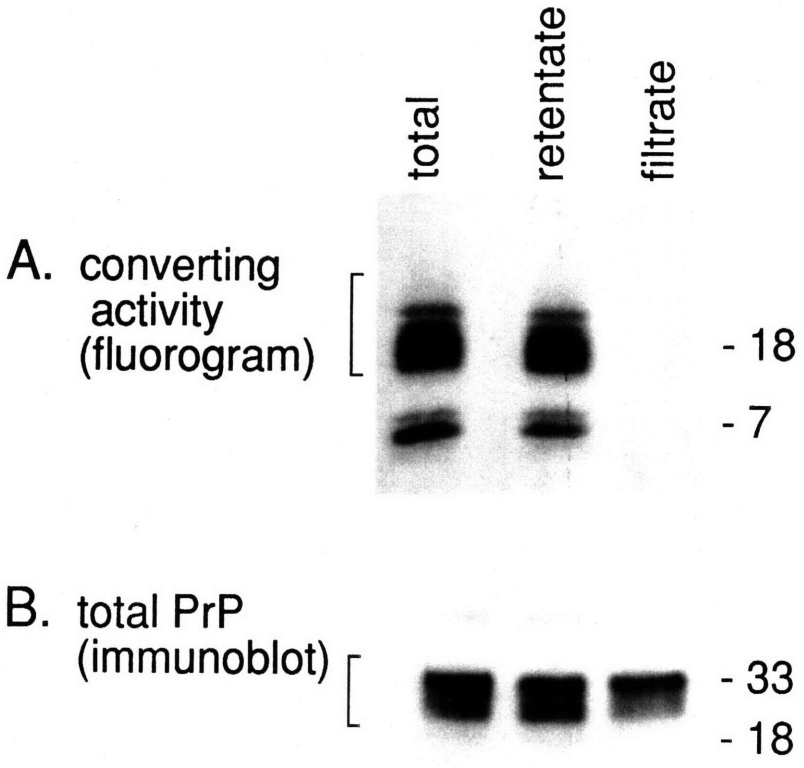

total
retentate
filtrate
A. converting activity (fluorogram)
- 18
- 7
B. total PrP (immunoblot)
- 33
- 18

Fig.3.7

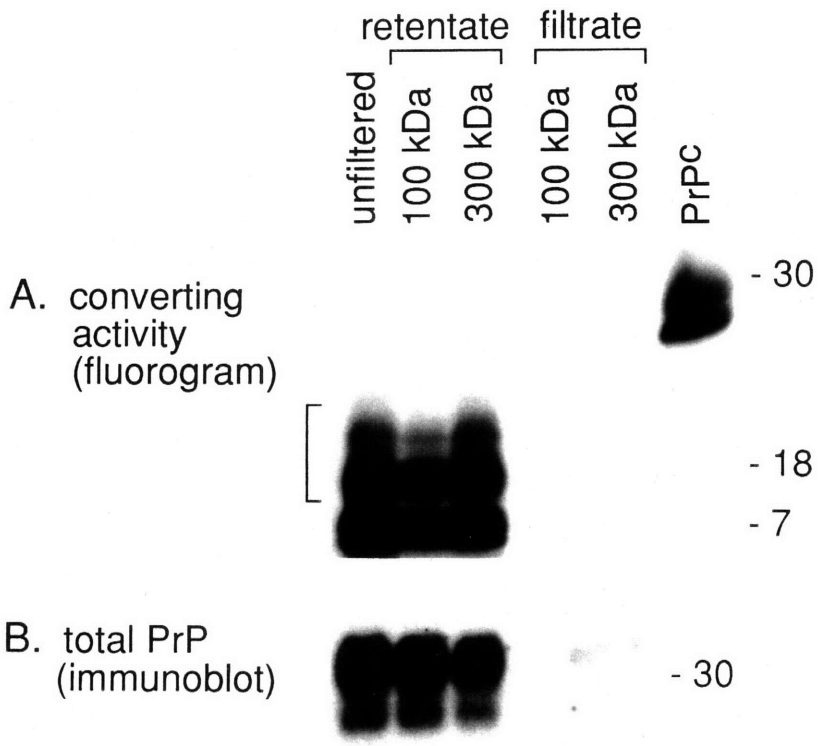
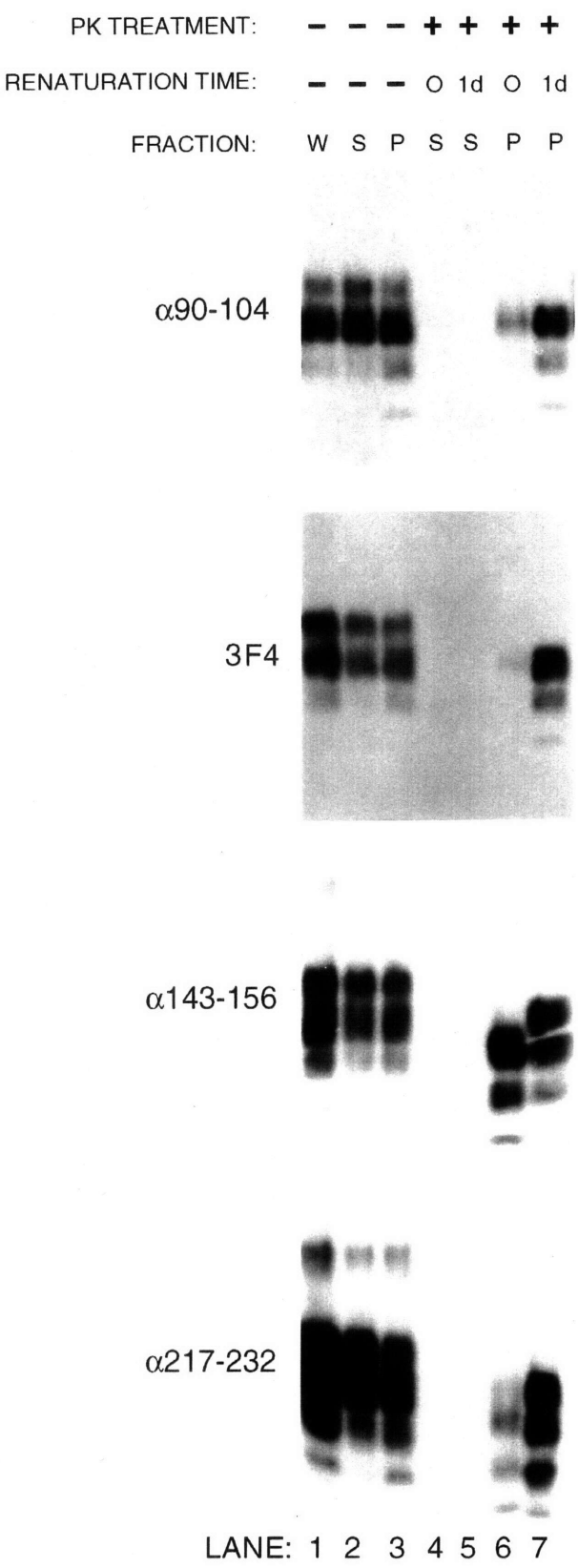

retentate
filtrate
unfiltered
100 kDa
300 kDa
100 kDa
300 kDa
PrPc
A. converting activity (fluorogram)
- 30
- 18
- 7
B. total PrP (immunoblot)
- 30

Fig.3.8


PK TREATMENT: - - - + + + +
RENATURATION TIME: - - - O 1d O 1d
FRACTION: W S P S S P P
α90-104
3F4
α143-156
α217-232
LANE: 1 2 3 4 5 6 7

Experimental

PrP^{Sc}

PrP^{Sc} was purified from the brains of hamsters infected with the 263K strain of scrapie as described in chapter 2 and was sonicated into 0.5-1% sulfobetaine 3-14 (SB) in phosphate buffered saline (20 mM sodium phosphate and 130 mM NaCl, pH 7.4) and stored at -20° C prior to use.

In some experiments, PrP^{Sc} was treated with PK prior to fractionation as follows. PrP^{Sc} at a concentration of 200 µg/mL in 10% (w/v) NaCl, 1% SB in TEND (10 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, and 130 mM NaCl, pH 8.3) was treated with 25 µg/mL PK for 1 hr at 37° C. The reaction was stopped by adding aprotinin, leupeptin, and Pefabloc to .01 µM, 1 µM, and 0.1 mM, respectively. The PrP^{Sc} was centrifuged for 15 minutes at 12,000 x g at 4° C and the supernatant removed. The pellet was sonicated into 0.1% N-lauroylsarcosine, .01 µM aprotinin, and 1 µM leupeptin. The PrP^{Sc} was centrifuged as before and the pellet sonicated into 1% SB and PBS and stored at -20° C until use.

Conversion of PrP^{C}

Conversion experiments were done very similarly to those for chapter 2. 2 µL of fractions to be tested for converting activity were added to reactions. Incubation times at 37° C ranged from 2-22 hours. Further dilution, PK treatment and SDS-PAGE were performed as stated in chapter 2. Besides using fluorography as mentioned in chapter 2, a Phosphorimager (Molecular Dynamics) was used to quantify ^{35}S in specific bands. The amount of converted ^{35}S-PrP GPI^{-} after PK treatment in the 17-21 kDa range was compared with known quantities of ^{35}S in non PK treated ^{35}S-PrP^{C} in the 24-28 kDa range. In this way percent conversion efficiencies were calculated.

Centrifugation of PrP^{Sc}

For the simple centrifugation of GdnHCl-treated PrP^{Sc} (as in Fig. 3.3), 50 µL of PrP^{Sc} pretreated at 1 mg/mL in 3 M GdnHCl for 5 hr at 37° C was centrifuged at 70,000 rpm for 50 min at 20° C in a Beckman TL100.1 rotor. The supernatant was collected and the pellet was resuspended by cuphorn sonication into 50 µL of fresh 3 M GdnHCl. Aliquots of each fraction were assayed for converting activity in the cell-free conversion reaction described above and for total PrP content by immunoblotting as described in chapter 2.

Sedimentation velocity gradients were performed by layering 20 µL of a given PrP^{Sc} preparation (preincubated at 37° C for 2-18 hr in 2.5 M GdnHCl) over 40 µL of a solution with identical GdnHCl concentration and 5% (w/v) sucrose in a Beckman 7 x 20 mm polycarbonate centrifuge tube. The tubes were spun to give the designated maximum g-force for 30 minutes at 20° C in a Beckman TLS 55 swinging bucket rotor using the slowest acceleration and deceleration settings. The gradients were fractionated as shown in Fig. 3.4 and the pellet was sonicated into an additional 20 µL of solution. Equal aliquots of the fractions were assayed for converting activity and for total PrP content by immunoblotting. Molecular weight standard proteins were dissolved in 2.5 M GdnHCl at approximately 1 mg/mL and fractionated on gradients exactly as was PrP^{Sc}. The concentrations of the molecular weight standards in the fractions was determined by SDS-PAGE with silver staining, except in the cases of thyroglobulin and blue dextran. The latter two were too large to enter the SDS-PAGE gels so they were run on separate gradients and their concentrations in the fractions were determined by absorbance at 280 nm and 625 nm, respectively.

Estimation of sedimentation coefficients

The sedimentation coefficient (s in Svedberg units, *S*) of particles expected to be 90% cleared from the sedimentation velocity gradient sample zones within

the time of centrifugation (t in hr) was estimated using the following equation from the Beckman centrifuge manual:

$$s_{obs} = k/t$$

where k is the clearing factor for the sample zone given by

$$k = 253303 \ln (r_{max}/r_{min}) / (RPM/1000)^2$$

where r_{max} and r_{min} are the maximum and minimum radii, respectively, of the sample zone within the spinning rotor and RPM is the revolutions per minute of the rotor.

Estimates of the $s_{20,w}$ values for the particles was obtained from s_{obs} using equation (2) of Prusiner, et. al.[29] to correct for the difference in density between 2.5 M GdnHCl and water. No correction for a difference in viscosity was made.

Ultrafiltration of PrP^{Sc}

Millipore Ultrafree-MC polysulfone membrane 100,000 and 300,000 NMWL filter units were used according to the manufacturer's instructions. PrP^{Sc} to be filtered was first incubated in 3 M GdnHCl at 37° C at 1 mg/mL for 2-5 hr. 50 µL of the PrP^{Sc} solution was loaded onto the filter and then centrifuged at 1-2,000 rpm until about half of the solution had been filtered. The retentate fractions were then diluted back to 50 µL with 3 M GdnHCl (the filtrates were left neat) and 2 µL of each was immediately added to a conversion reaction to assess converting activity. Equivalent parts of each fraction were also diluted and immunoblotted to determine the total PrP content. After an initial protease treatment, PK-treated PrP^{Sc} was filtered and assayed identically to non-protease treated PrP^{Sc}.

REFERENCES FOR CHAPTER 3

1. McKinley, M.P., Bolton, D.C. & Prusiner, S.B. *Cell* **35**, 57-62 (1983).
2. Caughey, B., Ernst, D. & Race, R.E. *J. Virol.* **67**, 6270-6272 (1993).
3. Prusiner, S.B. *Science* **252**, 1515-1522 (1991).
4. Griffith, J.S. *Nature* **215**, 1043-1044 (1967).
5. Bolton, D.C. & Bendheim, P.E. in *Novel Infectious Agents and the Central Nervous System* (eds. Bock, G. & Marsh, J.) 164-181 (John Wiley and Sons, Chichester, 1988).
6. Brown, P., Goldfarb, L.G. & Gajdusek, D.C. *The Lancet* **337**, 1019-1022 (1991).
7. Jarrett, J.T. & Lansbury, P.T., Jr. *Cell* **73**, 1055-1058 (1993).
8. Safar, J., Roller, P.P., Gajdusek, D.C. & Gibbs, C.J. *J. Biol. Chem.* **268**, 20276-20284 (1993).
9. Manuelidis, L., Sklaviadis, T., Akowitz, A. & Fritch, W. *Proc. Natl. Acad. Sci. USA* **92**, 5124-5128 (1995).
10. Oesch, B., Westaway, D., Wälchli, M., McKinley, M.P., Kent, S.B.H., Aebersold, R., Barry, R.A., Tempst, P., Teplow, B.B., Hood, L.E., Prusiner, S.B. & Weissmann, C. *Cell* **40**, 735-746 (1985).
11. Hope, J., Morton, L.J.D., Farquhar, C.F., Multhaup, G., Beyreuther, K. & Kimberlin, R.H. *The EMBO J.* **5**, 2591-2597 (1986).
12. Gaskin, F. & Canlor, C.R. *J. Mol. Biol.* **89**, 737-758 (1974).
13. Andreu, J.M. & Timasheff, S.N. *Methods Enzymol.* **130**, 47-59 (1986).
14. Asakura, S. *Adv. Biophys.* **1**, 99-155 (1970).
15. Eaton, W.A. & Hofrichter, J. *Adv. Protein Chem.* **40**, 63-279 (1990).
16. Murray, A.J., Lewis, S.J., Barclay, A.N. & Brady, R.L. *Proc. Natl. Acad. Sci. USA* **92**, 7337-7341 (1995).
17. Caughey, B.W., Dong, A., Bhat, K.S., Ernst, D., Hayes, S.F. & Caughey, W.S. *Biochemistry* **30**, 7672-7680 (1991).
18. Snow, A.D., Wight, T.N., Nochlin, D., Koike, Y., Kimata, K., Dearmond, S.J. & Prusiner, S.B. *Lab. Invest.* **63**, 601-611 (1990).
19. Caughey, B., Brown, K., Raymond, G.J., Katzenstein, G.E. & Thresher, W. *J. Virol.* **60**, 2135-2141 (1994).
20. Aiken, J.M. & Marsh, R.F. *Microbiol. Rev.* **54**, 242-246 (1990).
21. Riesner, D. *Sem. Virol.* **2**, 215-226 (1991).
22. Gabizon, R., McKinley, M.P., Groth, D.F., Kenaga, L. & Prusiner, S. B. *J. Biol. Chem.* **263**, 4950-4955 (1988).
23. Bellinger-Kawahara, C.G., Kempner, E., Grath, D., Gabizon, R. & Prusiner, S.B. *Virology* **164**, 537-541 (1988).
24. Safar, J., Wang, W., Padgett, M.P., Ceroni, M., Piccardo, P., Zopf, D., Gajdusek, D.C. & Gibbs, J.P.T. *Proc. Natl. Acad. Sci. USA* **87**, 6373-6377 (1990).
25. Gabizon, R., Mckinley, M.P. & Prusiner, S.B. *Proc. Natl. Acad. Sci. USA* **84**, 4017-4021 (1987).
26. Kempner, E.S. & Miller, J.H. *Science* **222**, 586-589 (1983).

27. Harmon, J.T., Nielson, T.B. & Kempner, E.S. *Methods Enzymol.* **117**, 65-94 (1985).
28. Harmon, J.T., Kahn, C.R., Kempner, E.S. & Schlegel, W. *J. Biol. Chem.* **255**, 3412-3419 (1980).
29. Prusiner, S.B., Hadlow, W.J., Eklund, C.M., Race, R.E. & Cochran, S.P. *Biochemistry* **17**, 4987-4992 (1978).

Chapter 4
Species specificity in the cell-free conversion of PrP^C to protease-resistant forms: a model for the species barrier

The barrier to interspecies transmission is an important phenomenon associated with the TSEs. If a specific interspecies transmission is possible, then a large increase in the incubation time of the disease is usually observed. In some cases, interspecies transmission is not observed. If an initial interspecies transmission was possible, on subsequent passages of the agent into the same species the barrier decreases. For instance, the incubation time of Chandler strain mouse scrapie is about 120 days into mice and about 380 days into hamsters.[1, 2] In contrast, the incubation time of the 263K hamster strain into hamsters is about 60 days, but the disease is not transmitted to mice (no disease after a two year observation period).[3] Extended incubation time in one direction is observed and a total barrier to disease in the other. Many other examples of such barriers have been noted.

As mentioned in chapter 1, in 1985 cows began getting sick with a scrapie-like disease in England. The disease, known as bovine spongiform encephalopathy (BSE), grew to epidemic proportions. While not known for certain, this outbreak may have originated as sheep scrapie. An endemic sheep scrapie exists in England (and the U.S.) and sheep meat and bone meal had been used as feed supplements for cattle for many years. In 1981, a solvent extraction and superheated steam treatment of the meal was discontinued. One theory of the origin of BSE is that eliminating these processing steps allowed sheep scrapie to infect cows through the feed. The only firmly established method of BSE

transmission to other cows has been through ingestion of contaminated meal.[4, 5] The expense of dead cattle was not the only problem for the British cattle industry. The fear of human susceptibility to BSE arose. The oral infection route being established, could humans get BSE from eating infected beef? The answer to that is a qualified yes. A new strain of CJD has been identified that is possibly caused from BSE infection in humans.[6] This new strain has different pathology from other CJD strains and has PrP plaques that look similar to those seen in kuru. The U.S. and Germany have banned the import of British beef since 1989 and 1990 respectively. Recently at least 22 other countries have also banned British beef causing economic hardship to Britain's multibillion dollar cattle industry. These bans will probably remain for some time as the risk of human transmission is studied. British consumption of beef has understandably declined sharply. The situation must be grave when even McDonald's restaurants in Great Britain have stopped using British beef.

In 1988 the British government banned the use of ruminant-derived feed supplements and now the number of new cases of BSE is declining. If there is no other method of transmission then the number should continue to drop. It is not known if BSE will become endemic to cattle the way scrapie is to sheep. Another possibility is that BSE was endemic to cattle but that changes in rendering allowed for the increased spread of the disease. Sheep scrapie has been a controllable problem. It spreads from animal to animal in a flock or from mother to unborn offspring. Sheep scrapie has been known for hundreds of years and has not been seen to be infectious to man based on epidemiological studies.

The cell-free conversion system had an obvious application in determining if the species barrier would apply *in vitro*. Not only can the cause of the barrier be studied, but the question of human infection by BSE can be probed.

Conversion of MoPrP^{C} to a resistant form by incubation with a MoPrP^{Sc} preparation

All previous experiments used 263K $HaPrP^{Sc}$ to convert different types of $HaPrP^{C}$ to the protease-resistant form. Experiments were then done to see if mouse PrP would work in this conversion system. To begin, a source of $MoPrP^{Sc}$ and ^{35}S-$MoPrP^{C}$ was needed. $MoPrP^{Sc}$ was purified from Chandler scrapie infected mouse brains using the $HaPrP^{Sc}$ purification procedure. A mouse cell line that produced wild type $MoPrP^{C}$ was used as that source. The $MoPrP^{C}$ was immunoprecipitated as $HaPrP^{C}$ had been except that a different antibody was used.

For use in a conversion reaction, the $MoPrP^{C}$ was eluted from protein A sepharose in 3 M GdnHCl and mixed with $MoPrP^{Sc}$ that had been treated overnight in 3 M GdnHCl. TN buffer was used to dilute the concentration of GdnHCl to 0.75 M and the concentrations of proteins were the same as that used for the hamster conversion. Protease-resistant PrP seemed to be produced whether $MoPrP^{Sc}$ was added or not. This protease-resistant material was also present in 2 minute incubation samples. This result was reproducible and it was concluded that 3 M GdnHCl trapped $MoPrP^{C}$ in an "abortive conformation" that the presence of $MoPrP^{Sc}$ did not affect. No similar effect was ever seen using $HaPrP^{C}$. It is not known if that material would be infectious.

To get around this "abortive conformation" the $MoPrP^{C}$ was eluted in higher concentrations of GdnHCl. Until the immunoprecipitated $MoPrP^{C}$ was protease sensitive, experiments to convert it into a protease resistant form would not work. Eluting the $MoPrP^{C}$ in 7.5 M GdnHCl was effective in making it properly PK sensitive. With that knowledge, the conversion experiment could be attempted again. The $MoPrP^{C}$ in 7.5 M GdnHCl was diluted with TN and then the $MoPrP^{Sc}$ in 3 M was added so it did not experience higher GdnHCl concentration. The protein concentrations were reduced because of extra

dilution to get to 0.75 M GdnHCl, but after a two day incubation at 37° C and PK treatment, protease resistant ^{35}S-MoPrP bands were observed.

The PK-resistant bands observed were 25-30 kDa, 19-21 kDa, and 15 kDa (Fig. 4.1). These different bands were probably due to the variable glycosylation of the MoPrP^{C}. As with the hamster conversion, the presence of the bands required the presence of the MoPrP^{Sc} preparation during incubation at 37° C. The MoPrP^{C} was initially converted in 0.75 M GdnHCl, but an increase in GdnHCl during conversion yielded a higher percent of conversion. GdnHCl concentrations from 1 to 6 M were used during the incubation to optimize the conversion efficiency. Incubation in 2 M GdnHCl gave the most efficient conversion although 1 and 3 M produced lesser amounts of identical bands (Fig. 4.2). This was very different from the hamster system where an incubation at 2 M GdnHCl produced very little protease resistant material (Fig. 4.3). These experiments showed that other species PrPs could be used in this conversion system, but that there were differences in how to achieve a maximum conversion.

One somewhat disturbing aspect of the conversion using Chandler strain MoPrP^{Sc} was that the products generated after PK treatment are reduced about 10-12 kDa. PK treatment of hamster and mouse brain derived PrP^{Sc} reduced its mass 6-7 kDa as was also seen with the cell-free conversion of ^{35}S-HaPrP^{C}. The formation of these bands was dependent upon the presence of MoPrP^{Sc}, but this increased amount of PK cleavage indicated that ^{35}S-MoPrP^{Sc} may not have been fully formed. It is possible that a PK-resistant conformation of PrP was generated that was different from MoPrP^{Sc} or intermediate between MoPrP^{C} and MoPrP^{Sc}. However, when a different strain of MoPrP^{Sc} called 87V was later used, the PK-resistant bands were reduced by the expected 6-7 kDa (data not shown). This could be another example of strain differences seen using this cell-

free conversion system covered in chapter 5. Other conversions using 87V are in progress. Again, the infectivity of PK-resistant MoPrP compared with brain derived $MoPrP^{Sc}$ could not be determined because of the vast amount of pre-existing mouse infectivity.

Conversion experiments using combinations of HaPrP and MoPrP

With homogenous combinations of Ha and MoPrPs working, conversion reactions were done using different species. ^{35}S-$MoPrP^{C}$ incubated with $HaPrP^{Sc}$ and treated as before for the optimal hamster or mouse conversion yielded no PK-resistant material (Fig. 4.1, compare lanes 3 and 4). To learn if glycosylation was interfering with the interspecies conversion, tunicamycin was used during the labeling to prevent Asn glycosylation. A 25 kDa unglycosylated $MoPrP^{C}$ resulted that was used in the conversion with Mo and $HaPrP^{Sc}$. Incubation with $MoPrP^{Sc}$ resulted in three bands between 12 and 15 kDa (Fig. 4.1, lane 8). Incubation with $HaPrP^{Sc}$ resulted in little detectable conversion (lane 7), which showed that more than glycosylation was responsible for this *in vitro* species barrier.

The conversion reaction using ^{35}S-$HaPrP^{C}$ expressed with and without the GPI anchor was performed with $MoPrP^{Sc}$. This combination yielded very different results than the previous one. Figure 4.3 is a fluorogram that shows the results of the incubation of the two ^{35}S-$HaPrP^{C}$ constructs with $MoPrP^{Sc}$. $MoPrP^{Sc}$ was able to generate resistant bands with either construct, but they were different from those generated by $HaPrP^{Sc}$. As an example, in lane 3 the most intense band after incubation with $MoPrP^{Sc}$ was 18 kDa compared to lane 2 which shows a 25 kDa band from $HaPrP^{Sc}$. Differences were also seen with the GPI^{-} $HaPrP^{C}$ construct shown in lanes 7 and 8 that depended on whether Ha or $MoPrP^{Sc}$ was present during the incubation.

The reactions were incubated in different concentrations of GdnHCl to see if these species specific differences would continue. The mouse and hamster reactions were performed at 2 M GdnHCl (the optimum for mouse-mouse conversion) as well as 0.75 M (Fig. 4.3). The species dependent size differences were similar in either GdnHCl concentration, but the extent of conversion was different. Changing the concentration of GdnHCl during incubation did not eliminate the species specificity.

^{35}S-HaPrP^{C} was eluted in either 3 or 7.5 M GdnHCl before being used in the conversion reactions. The products of 3 and 7.5 M pretreated HaPrP^{C} with HaPrP^{Sc} were directly compared. No difference in their abilities to be converted under otherwise identical conditions was seen (data not shown), providing evidence that any additional denaturing done by the increased GdnHCl was not important. For reasons mentioned earlier, all ^{35}S-MoPrP^{C} was pretreated in 7.5 M GdnHCl. Extra denaturation done by increased denaturant concentration was only important when ^{35}S-MoPrP^{C} was used. It appeared that once the PrP^{C} was denatured to a certain extent, the only factor determining its conversion was the species of PrP^{Sc} it was incubated with.

Conversion experiments of mouse and hamster chimeras with PrP^{Sc}

Experiments were done to determine if a specific region of PrP was responsible for the species specificity of the conversion to a protease-resistant form. Specific PrP sequence differences affecting the conversion would point to direct PrP^{C}-PrP^{Sc} interactions. At RML, chimeric PrPs were produced that included portions of mouse and hamster sequence for use in cell culture conversion studies. To be used in the cell-free conversion system they just had to be immunoprecipitated as was done with the other constructs. As mentioned before, there are 16 sequence differences between mouse and hamster PrP with

two of the differences being missing residues. Figure 4.4 is a map of the constructs that were used in conversion reactions with Ha or MoPrP^{Sc}.

Conversion experiments with the chimeras were done very similarly to those described earlier. The chimeras and mouse and hamster PrP^{C} were all treated in 7.5 M GdnHCl and mixed with MoPrP^{Sc} or HaPrP^{Sc} that had been treated in 3 M GdnHCl. One difference was that the incubation was in 1.25 M GdnHCl which allowed conversion by Mo or HaPrP^{Sc} but was not optimal for either one. The results from similar experiments in 0.75 and 2 M only differed from 1.25 M GdnHCl in the extent of conversion in either case. All constructs incubated with MoPrP^{Sc} produced PK-resistant bands (Fig. 4.5). Some minor variation in the size of the major bands produced was seen, but this can be explained by different levels of glycosylation from the different expressing cell types. However, after incubation with HaPrP^{Sc}, only Mo/Ha 33, Mo/Ha 67, and HaPrP^{C} yielded obvious PK-resistant material. These products were up to 32 kDa in mass and the pattern was like that usually seen when PrP^{Sc} was treated with PK. The HaPrP^{C} GPI^{-} construct converted as expected. Its banding pattern was always different from the other constructs because of less glycosylation and the missing membrane anchor.

The readily apparent feature of those constructs that significantly converted in the presence of HaPrP^{Sc} was that the central PrP region was of the hamster sequence. The difference between hamster and mouse sequences in that region is only three amino acid residues. Differences in other regions did not control if conversion occurred.

What is the cause of this in vitro *species barrier?*

The species barrier is a virus-like characteristic of the TSEs and consequently, many have argued that this is evidence a mutable nucleic acid genome is part of the infectious agent. However, no virus or scrapie-specific

nucleic acid has been found to date. The argument can be made from this study that it is caused through protein-protein interactions. The species barriers known *in vivo* have been duplicated in this cell-free system. As before, some doubt remains in interpretation of these results because the purity of the PrP^{Sc} used is not 100% and the presence of some important contaminant can not be ruled out. Regardless of the possible presence of some important contaminant, the species specificity in conversion of PrP^{C} to the protease-resistant form depends at least in part upon the PrP sequences in the reaction.

A central observation here is that $MoPrP^{Sc}$ and $HaPrP^{Sc}$ converted identical $HaPrP^{C}$ into products that had different PK sensitivities. One interpretation of this result is that the different PrP^{Sc}s induced different conformations in the PrP^{C}. The different conformations caused it to be cleaved differently by PK which would explain the different sized bands seen. Aggregates of different PrP^{Sc} could lead to the formation of different conformations in converted PrP^{C} through a seeded polymerization mechanism, for instance.

This *in vitro* species barrier seems to correlate with the *in vivo* data on transmission of 263K and Chandler scrapie between mice and hamsters. Hamster 263K scrapie is not transmissible to mice. Correspondingly, there was no conversion of $MoPrP^{C}$ to the resistant form using $HaPrP^{Sc}$. $MoPrP^{C}$ is converted to $MoPrP^{Sc}$ when mice get scrapie. Chandler scrapie is transmissible to hamsters but an extended incubation time is required. The fact that $MoPrP^{Sc}$ converts $HaPrP^{C}$ to a form with a different protease sensitivity than $HaPrP^{Sc}$ may account for this extension. The conformation of $HaPrP^{C}$ induced by $MoPrP^{Sc}$ may be less pathogenic than generated by $HaPrP^{Sc}$, accounting for the delayed onset seen *in vivo*. If PrP^{C} converting to a protease-resistant form is

equivalent to the pathology of the disease, this data correlates with the biological data available.

A difference of three amino acids was sufficient to stop the conversion to a resistant form. Mo/Ha 40 and Mo/Ha 67 only differ at residues 139, 155, and 170. The changes in the sequence going from the hamster to the mouse sequence (Met 139-Ile, Asn 155-Tyr, Asn 170-Ser) are conservative and occur in a hydrophilic region of PrP. These residues were essential if a protease resistant form was going to be induced with $HaPrP^{Sc}$. Since $MoPrP^{Sc}$ converted all Mo and $HaPrP^{C}$ to resistant forms, it is not surprising that it converted all chimeras as well. Because these residues occur in a hydrophilic region, it is reasonable to assume they be at the surface of PrP^{C} and could be available to react with PrP^{Sc} during the conversion. Experiments are in progress using $MoPrP^{C}$ with only residues 139, 155, or 170 changed to the hamster version.

If the 135-175 region is responsible for the species barriers seen in the TSEs then it is of interest to see how these regions compare between other species. Between bovine and ovine PrP there are differences at 143, 155, and 168, three in all as is the case between hamster and mouse. Human and bovine are different at 138, 166, and 168, while human and ovine have differences at 138, 143, 155, 166, and 168. The apparent lack of transmission of scrapie from sheep to humans could be due to the extra differences in the 135-175 region, but cell culture studies have demonstrated that one differing residue (138) is sufficient to prevent conversion to PrP^{Sc} using mouse and hamster sequences. Perhaps cows might be more susceptible to sheep scrapie because there is no difference at 138. The human sequence differs from ovine and bovine at residue 138 which hopefully lowers human susceptibility to BSE. Clearly more than just the number of sequence differences is important. The direction of transmission is also important as hamster 263K scrapie will not cause disease in mice, but Chandler

mouse scrapie will cause disease in hamsters. Sheep scrapie might not be infectious to humans, but passed through cows as BSE it might be. Cell-free conversion experiments are currently in progress using human, bovine, and ovine PrP^{C}s and PrP^{Sc}. These experiments will determine if the sequences are compatible using *in vitro* conditions. However, the exact correlation between *in vitro* compatibility and infectivity is unclear.

For PrP^{Sc} to be the infectious agent in scrapie it has to be capable of explaining the species barriers that are encountered. According to this study,[7] except for the possibility of a biological contaminant that copurifies with PrP^{Sc}, direct protein-protein interactions can explain species barriers. Different species PrPs have various sequence differences which could be expected to vary their structures enough to change compatibilities on a molecular level. The more compatible that the sequences are, the more likely that a TSE will be transmissible between them.

Figure 4.1 Conversion of glycosylated (Mo) or tunicamycin-treated (+tun) ^{35}S-$MoPrP^{C}$ to PK-resistant forms in the presence of $MoPrP^{Sc}$ or $HaPrP^{Sc}$. Without PK treatment, the glycosylated ^{35}S-$MoPrP^{C}$ precursor consisted of 25-40 kDa bands (lane 1) and the unglycosylated form migrated at approximately 25 kDa (lane 5). All PrP^{C} used here was eluted from protein A sepharose in 7.5 M GdnHCl and incubated in 2 M GdnHCl. PK-treated lanes contained twice the reaction equivalents loaded into the non PK-treated lanes. Molecular weight markers are indicated in kDa at the left.

Figure 4.2 The effect of GdnHCl concentration on conversion of $MoPrP^{C}$ to a resistant form using $MoPrP^{Sc}$. Lane 1 was non PK-treated ^{35}S-$MoPrP^{C}$ precursor. Lanes 2-6 were PK-treated ^{35}S-$MoPrP^{C}$ after incubation with $MoPrP^{Sc}$ at the indicated GdnHCl. Notice how there was no conversion in 4.5 or 6 M GdnHCl and that the only difference in the 1, 2, and 3 M lanes was the intensity of the signal. The concentration of GdnHCl was made 1 M for all samples before adding PK. Lane 1 contained 7 times fewer reaction equivalent than the other lanes. Molecular weight markers are indicated in kilodaltons at the left.

Figure 4.3 Conversion of $HaPrP^{C}$ to resistant forms using $HaPrP^{Sc}$ and $MoPrP^{Sc}$. Without PK treatment, the ^{35}S-$HaPrP^{C}$ precursor consisted of 25-40 kDa bands

and a 60 kDa dimer (lane 1) and the ^{35}S-HaPrP^{C} GPI^{-} construct consisted predominantly of an unglycosylated (24 kDa) form (lane 6). All HaPrP^{C} used here was eluted from protein A sepharose in 3 M GdnHCl and incubated in 0.75 or 2 M GdnHCl. All PK-treated lanes contained 7 times the reaction equivalents loaded into the non PK-treated lanes. Molecular weight markers are indicated in kDa at the left.

Figure 4.4 Map of the mouse, hamster, and chimeric PrP^{C} forms used in Figure 4.5. Shaded areas indicate regions that were derived from hamster DNA and open areas were from mouse. Tick marks indicate where amino acid residues differ between mouse and hamster PrP and the ticks over the bars designate the presence of a hamster residue in that position. 16 differences are indicated between hamster and mouse sequences. NaeI and BstEII refer to the sites where the different DNAs were joined. The five numbered residues were present in all constructs that converted to resistant forms with HaPrP^{Sc}. A plus to the right of a bar signifies that the construct converted to a resistant form after incubation with the indicated PrP^{Sc}. Notice that all constructs that converted with HaPrP^{Sc} had hamster residues (methionines) at residues 109 and 112 (partially defining the 3F4 epitope) but these residues were not sufficient for conversion with HaPrP^{Sc} as indicated by the lack of conversion of Mo-3F4.

Figure 4.5 The conversion of chimeric mouse/hamster PrP^{C}s to PK-resistant forms in the presence of HaPrP^{Sc} or MoPrP^{Sc}. The constructs were pretreated with 7.5 M GdnHCl and the incubation with PrP^{Sc} was at 1.25 M GdnHCl. Lanes 1-7 illustrate the untreated ^{35}S-PrP^{C} constructs. Lanes 8-21 were the constructs after incubation with PrP^{Sc} and PK treatment. Molecular weight markers are indicated in kDa at the left.

Fig.4.1

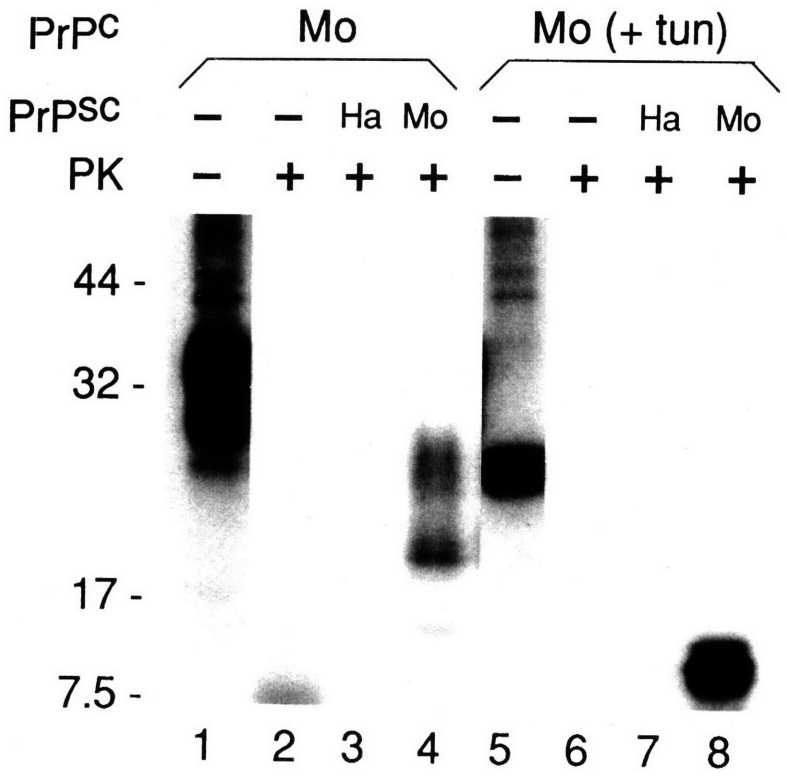


Fig.4.2

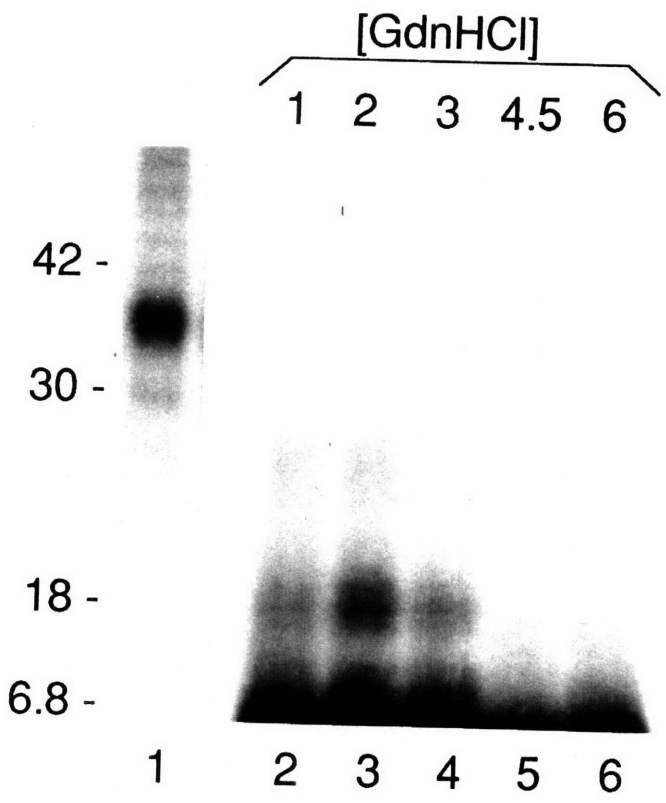
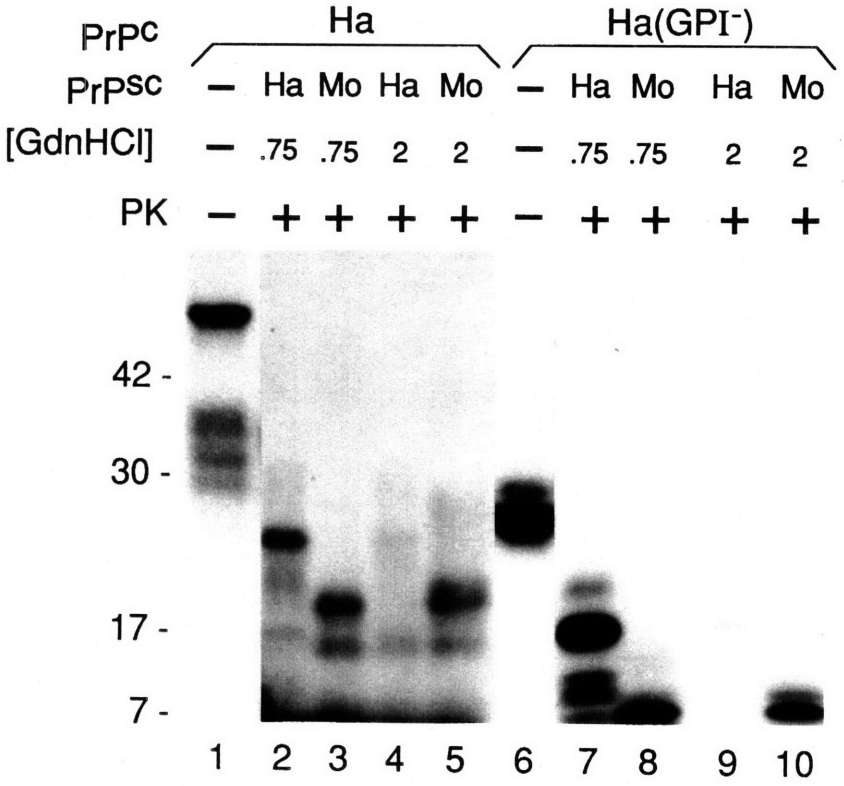


Fig.4.3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Ha
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Mo
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Ha
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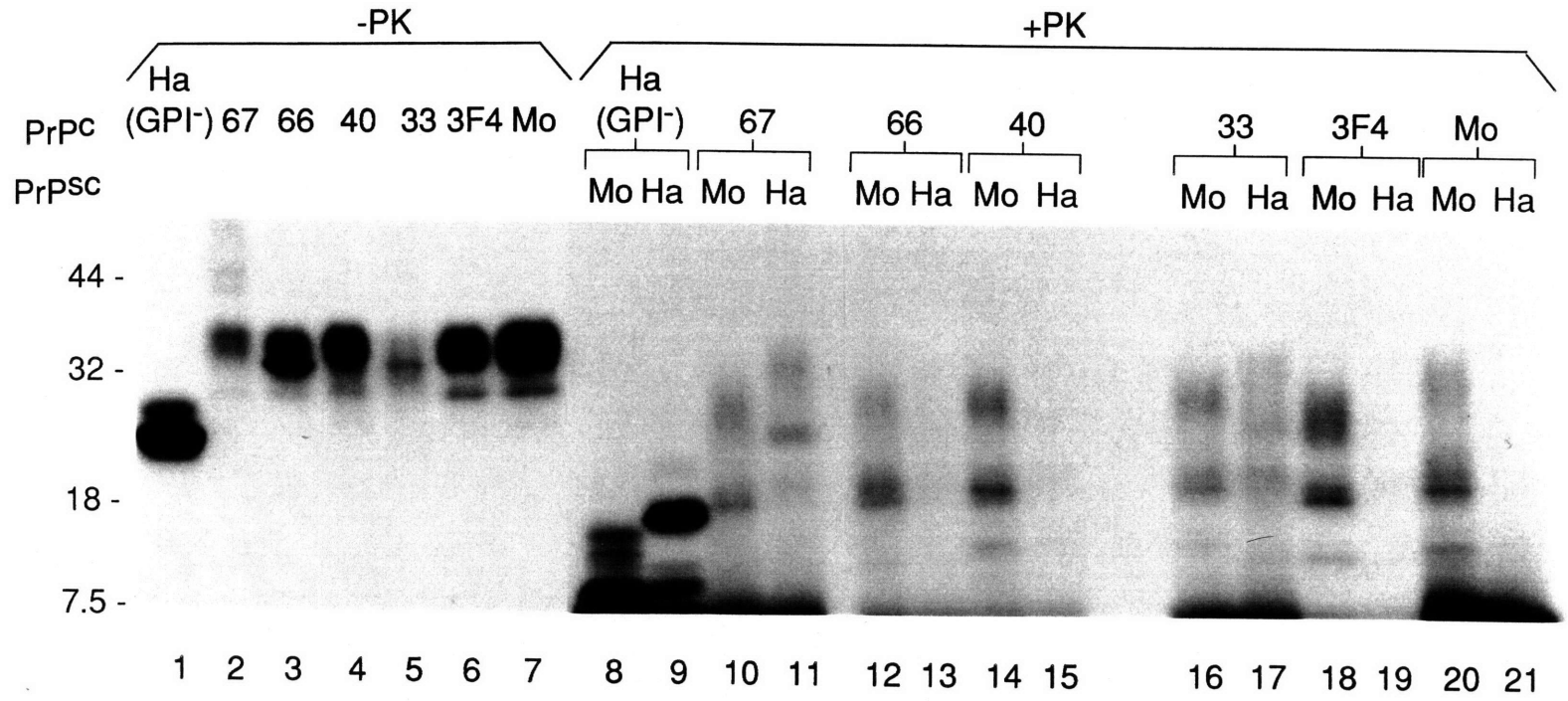
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3F4
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7.5 -
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21

Fig.4.5

Experimental

Preparation of $MoPrP^{Sc}$

The $MoPrP^{Sc}$ was purified using the procedure for $HaPrP^{Sc}$ described in chapter 2. The source of $MoPrP^{Sc}$ was mouse brains infected with Chandler strain scrapie. The preparations were used directly from a suspension in 1% sulfobetaine 3-14 and phosphate buffered saline.

Formation of mouse/hamster chimeras

Mo-3F4, which is MoPrP containing the hamster reactive 3F4 antibody epitope (Met 109 and Met 112) and a unique Nae I site has been described.[8] Two mouse/hamster PrP chimeras, Mo/Ha 33 and Mo/Ha 40, were derived by utilizing the unique Nae I site within HaPrP and Mo-3F4 as described.[9, 10] Additional recombinants Mo/Ha 66 and Mo/Ha 67 were derived by PCR mutagenesis using standard techniques.

Recombinant PrP molecules were expressed in normal mouse neuroblastoma (MNB) cells using the pSFF retroviral expression system. Cells expressing the other recombinant PrP molecules were derived by limited dilution cloning of bulk cultures of MNB cells expressing recombinant PrP molecules according to published procedures.[9, 10]

Labeling of mouse and mouse/hamster chimeric PrP^{C}

Chimeric mouse/hamster PrP^{C} was labeled with ^{35}S methionine and cysteine and immunoprecipitated from lysed cells using 3F4, a mouse monoclonal antibody, which reacted with hamster PrP and all of the chimeric PrPs containing the 3F4 epitope. In the case of MoPrP, which lacked the 3F4 epitope, R30,[11] a rabbit polyclonal antiserum raised against PrP 90-104 was used. The PrP-antibody complexes are eluted from protein A sepharose in either 3 or

7.5 M GdnHCl. Except for the use of different antibodies and different GdnHCl concentrations during elution, the labeling is done as was stated in chapter 2.

Conversion of mouse and mouse/hamster chimeric PrP^Cs to protease-resistant forms

Conversion of these PrP^Cs to resistant forms was done with small changes on the basic reaction mentioned in chapter 2. $MoPrP^C$ was eluted in 7.5 M GdnHCl and mixed with $MoPrP^{Sc}$ and then diluted to 2M GdnHCl, the optimal concentration. After incubation and before PK treatment, the mixture was further diluted to 0.25 M GdnHCl using additional TN solution. The remainder of the procedure was done exactly as stated for conversion of $HaPrP^C$.

REFERENCES FOR CHAPTER 4

1. Kimberlin, R.H. & Walker, C.A. *J. Gen. Virol.* **34**, 295-304 (1977).
2. Kimberlin, R.H., Cole, S. & Walker, C.A. *J. Gen. Virol.* **68**, 1875-1881 (1987).
3. Kimberlin, R.H., Walker, C.A. & Fraser, H. *J. Gen. Virol.* **70**, 2017-2025 (1989).
4. Wilesmith, J.W. & Ryan, J.M.B. *Vet. Rec.* **130**, 491-492 (1992).
5. Wilesmith, J.W. & Ryan, J.M.B. *Vet. Rec.* (1993).
6. Will, R.G., Ironside, J.W., Zeidler, M., Cousens, S.N., Estibeiro, K., Alperovitch, A., Poser, S., Pocchiari, M., Hofman, A. & Smith, P.G. *Lancet* **347**, 921-925 (1996).
7. Kocisko, D.A., Priola, S.A., Raymond, G.J., Chesebro, B., Lansbury, P.T., Jr. & Caughey, B. *Proc. Natl. Acad. Sci. USA* **92**, 3923-3927 (1995).
8. Bolton, D.C., Seligman, S.J., Bablanian, G., Windsor, D., Scala, L.J., Kim, K.S., Chen, C.M.J., Kascsak, R.J. & Bendheim, P.E. *J. Virol.* **65**, 3667-3675 (1991).
9. Chesebro, B., Wehrly, K., Caughey, B., Nishio, J., Ernst, D. & Race, R. *Dev. Biol. Stand.* **80**, 131-140 (1993).
10. Priola, S.A., Caughey, B., Race, R.E. & Chesebro, B. *J. Virol.* **68**, 4873-4878 (1994).
11. Caughey, B., Raymond, G.J., Ernst, D. & Race, R.E. *J. Virol.* **65**, 6597-6603 (1991).

Chapter 5
Cell-free conversion of PrP into strain-specific protease resistant forms

Another virus-like characteristic of the TSEs is the existence of strains within the same species. Strains are identified through specific pathological features such as specific patterns of degeneration in the brain, different areas of the brain being affected, and the appearance of different clinical symptoms. Different strains are also noted for having different incubation times. The differences seen are analogous to variations in a disease caused by different strains of a virus. Many strains are stable when passaged between the same species of host and the pathology will not vary. No scrapie virus ever being identified, the question remains of what causes TSE strains. If PrP^{Sc} is the sole component of the infectious agent, then strains must be caused by propagated structural differences.[1, 2]

The propagation of strains can be tested in this cell-free conversion system. The problem is that while all different strains produce infectious PrP^{Sc}, in general, the pathology of newly formed infectivity would have to be monitored to know if a strain had been propagated. As was mentioned before, this system has too much pre-existing infectivity to determine if the strain characteristics had been passed. Strains were needed that produced PrP^{Sc} with a readily observable biochemical difference.

Two scrapie strains in Syrian golden hamsters that were suitable for use in the cell-free conversion system were named hyper (HY) and drowsy (DY).[1, 3] These strains originated from the inoculation of transmissible mink

encephalopathy infected brain homogenate into hamsters. As with other strains these have varying incubation times and clinical symptoms. However, the PrP^{Sc} from these strains has different sensitivities to PK cleavage and the N-terminal cleavage sites are different. A 1-2 kDa difference was seen after treatment with PK (Fig. 5.1**a**). Differences in the biochemical properties of other strains of PrP^{Sc} have been reported, but not as dramatic and reproducible as these. This difference could be interpreted to be due to different conformations. This presented an ideal situation for the cell-free system because if strain-specific N-terminal cleavage sites could be passed it would be readily observed without having to monitor infectivity.

Conversion experiments done using HY and DY PrP^{Sc}

HY and DY PrP^{Sc} for use in the cell-free conversion system was purified from infected hamster brains according to the procedure used for the Ha and $MoPrP^{Sc}$. The ^{35}S-$HaPrP^{C}$ used was the GPI^{-} form and it was immunoprecipitated to purify it as was done before. The reaction was performed as in earlier attempts by mixing the ^{35}S-$HaPrP^{C}$ with 3 M GdnHCl treated DY or HY PrP^{Sc} and then diluting to 0.75 M and incubating for two days. PK treatment and the rest of the procedure was done as before. The results of these conversions are shown in figure 5.1**b**. Similar differences in the truncation by PK of the converted material were present that depended upon the presence of a specific strain. ^{35}S-$HaPrP^{C}$ incubated with DY PrP^{Sc} was PK-resistant, but allowed an extra 1-2 kDa to be cleaved just as seen in DY PrP^{Sc} from infected brains. The major 24 kDa ^{35}S-PrP^{C} precursor was reduced in molecular weight to 16 or 17 kDa depending on which strain of PrP^{Sc} was present. As was demonstrated, incubation without the presence of any PrP^{Sc} prevented the formation of protease resistant material.

The efficiency of conversion of ^{35}S-PrP^C to a PK-resistant form was greater when incubated with HY PrP^{Sc} than DY PrP^{Sc}. When equal amounts of labeled PrP^C were incubated with equal amounts of HY or DY PrP^{Sc} much more protease resistant material was seen (Fig. 5.1**b**, compare lanes 1 and 2). A 16-fold increase in DY concentration relative to HY PrP^{Sc} resulted in approximately equal conversions based on the signal after PK treatment (lanes 3 and 4).

Another control was done to eliminate the possibility that more rigorous treatments with PK would further cleave the product of incubation with HY PrP^{Sc} to similar products as with DY PrP^{Sc}. Treatments of 0, 50, and 500 μg/mL PK were done on the conversion reaction mixture after incubation. As shown in Fig 5.1**c**, no formation of 16 kDa resistant bands occurred after incubation with HY PrP^{Sc} and extra PK treatment. The only difference was elimination of additional newly-converted labeled PrP from mixtures incubated with either PrP^{Sc}. The extra elimination was expected because long protease treatments (>24 hours) will eventually eliminate all PrP^{Sc}.

The conversion reaction was done with varying GdnHCl concentration during incubation to see if that affected strain differences. As is shown in figure 5.2, the extent of conversion was affected in different GdnHCl. No conversion occurred in 3 M, but it did in 1 or 2 M. Between 1 and 2 M only the extent of conversion was affected and the strain-specific differences remained. It does not appear that the differences were related to the GdnHCl concentration used in the reaction. The fact that strain-specific differences were maintained under a variety of conditions is evidence that they are due to stable differences between HY and DY PrP^{Sc}.

In vivo data correlates with the conversion observed using HY and DY PrP^{Sc}. There is a 100 fold greater concentration of infectivity in the HY infected brain than in the DY at the time of onset of disease, even though similar amounts

of PrP^{Sc} are present. In the cell-free system, approximately 16 fold more DY was needed to equal the conversion with HY. If accumulation of PrP^{Sc} is the major factor in disease progression, less infectivity per amount of PrP^{Sc} preparation correlates to lower levels of conversion in the cell-free system.

Conversion is enhanced by the presence of cationic detergents

Detergents added to the cell-free conversion reaction increased the percent of PK-resistant product formed. Cetyl pyridinium chloride (CPC), cetyl trimethyl ammonium bromide (CTAB), and triton X-100 below their critical micelle concentrations were included. 1.5 mM CPC or CTAB improved the extent of conversion (Fig. 5.3) to at least 30%. Compared to a reaction with no detergent, triton X-100 inhibited conversion. Decreasing the amount of CPC or CTAB decreased the enhancement and when less triton X-100 was present the inhibition was decreased. Controls were done that showed CPC alone cannot cause PrP to become protease-resistant and also that CPC does not inhibit PK (data not shown). CPC and CTAB are cationic detergents and CPC is actually an ingredient of mouthwash. Why they enhanced the reaction is unknown. It can be speculated that they help solubilize the reactants which boosted the conversion or mimic the membrane conditions that PrP experiences as it is bound to the cell surface. Triton X-100 is an anionic detergent and why it inhibited is again unknown.

CPC was added to the conversion using HY and DY PrP^{Sc} to see if the conversion would be enhanced and also if the strain differences would remain. 1.5 mM CPC improved the extent of conversion as much as 3.5 fold with HY and 14 fold with DY (Fig. 5.4). CPC also slightly altered the pattern of PK-resistant bands seen, but strain-specific differences were maintained.

PrP^{Sc} treated with PK causes conversion of PrP to the protease-resistant form

HY and DY PrP^{Sc} that had been pretreated with PK and then used in the conversion reaction as before yielded the same resistant bands as seen previously with strain differences being maintained (Fig 5.5). The treatment with PK prior to use in the conversion reaction resulted in a more pure PrP^{Sc} preparation; other minor protein impurities are largely eliminated. In the case of HY and DY PrP^{Sc}, the cleavage is at different sites but does not affect the strain differences. PK-treated PrP^{Sc} causing conversion is additional evidence that PrP interactions are sufficient for conversion to occur. A protein cofactor would have to copurify with PrP and also be protease-resistant. Of course potential non-protein cofactors would be unaffected.

When PK-treated PrP^{Sc} was used in the conversion system, the presence of PK must be taken into account. Trace amounts were likely to be present even after washing and pelleting steps. The conversion using PK-treated PrP^{Sc} worked poorly without CPC present (data not shown) and this could be because of residual PK digesting ^{35}S-PrP^{C} in the conversion mixture. It is possible that CPC sped up the reaction so digestion of the ^{35}S-PrP^{C} by residual PK was minimized.

Glycosylation does not account for the strain-specific differences in converted PrP^{C}

The major band of the ^{35}S-PrP^{C} GPI^{-} construct was a 24 kDa unglycosylated species. Some 28 kDa monoglycosylated PrP was present with the unglycosylated material. To make certain that glycosylation of the PrP^{C} was not a factor in strain differences, PrP^{C} from tunicamycin treated cells was immunoprecipitated as before. This unglycosylated ^{35}S-PrP^{C} GPI^{-} was used in the conversion reaction as stated above. The results of the conversion done with exclusively unglycosylated PrP^{C} indicated that the strain difference was maintained (Fig. 5.6). This experiment showed that the different forms were

derived from identical precursor material which consisted of a polypeptide of approximately 209 amino acids.

Evidence that protein alone can account for TSE strains

TSE strains are a virus-like property and have been difficult to explain using a protein-only hypothesis. Strains occur in the same species so there is no difference in primary PrP sequence as is the case in the species barriers. In this study[4] the identical 209 amino acid sequence of the PrP^{C} GPI^{-} construct was converted into different protease resistant forms through incubation with HY or DY PrP^{Sc} preparations. Because the precursor sequences were identical, it makes it likely that the differences were due to different tertiary or quartenary structure.

A precedented mechanism for PrP^{Sc} formation based on nucleated polymerization has been proposed.[5, 6] Using this model the strains can be reconciled with a protein only hypothesis of TSE infection (Fig. 5.7). In this scenario, scrapie strains are alternative conformations or different packing arrangements of similar conformations in PrP^{Sc} polymers, analogous to different crystal forms seen in the same protein. Although many proteins self-assemble into polymers by nucleation-dependent polymerization, this model of TSEs would be the first example of an infectious pathogen using only this mechanism for replication and strain variation. This data shows that the preexisting PrP^{Sc} can determine the structure of the newly formed PrP^{Sc} is consistent with this model. These results also show that the chemical conditions of the reaction can strongly affect the efficiency of the conversion. It is conceivable that diversity of the chemical or physiological conditions within the brain could determine the strain-specific tissue distribution of HY and DY PrP^{Sc} and the rate of PrP^{Sc} formation. These differences in PrP^{Sc} formation can determine the strain specific clinical and pathological features among scrapie diseases.

Figure 5.1 Strain-specific cell-free conversion of PrP^C to PK resistant PrP. **a**, Immunoblot of purified brain derived PrP^{Sc} from HY (H) and DY (D) scrapie infected hamsters digested with PK. Note the 1 kDa faster migration of DY PrP^{Sc} compared to HY PrP^{Sc}. **b**, 2 μg of HY and DY PrP^{Sc} (lanes 1 and 2), or 0.25 μg of HY PrP^{Sc} (lane 3) and 4 μg DY PrP^{Sc} (lane 4), were combined with approximately 60,000 cpm of ^{35}S-PrP^C in the conversion reactions. Lane 5 contains 10,000 cpm of ^{35}S-PrP^C. **c**, Cell-free conversion products digested with increasing amounts of PK. Conversion reaction ^{35}S-PrP products were analyzed by 15% SDS-PAGE and fluorography. The major recombinant ^{35}S-PrP^C had a molecular weight of 24 kDa (indicated by the arrowhead) which was smaller than the hamster brain derived PrP^C (33-35 kDa) because it lacked N-linked glycans and a GPI anchor. These differences between the sources of PrP^C can also explain why the banding pattern of the PK resistant ^{35}S-PrP in SDS-PAGE were not identical to the three PK resistant bands derived from hamster brain PrP^C. In some experiments, residual levels of the 24 kDa ^{35}S-PrP^C were present after PK digestion. Similar amounts of HY and DY PrP^{Sc} were present after PK digestion as determined by PrP immunoblots and silver stained SDS-PAGE (data not shown). Molecular weight markers are indicated at the tick marks.

Figure 5.2 The effects of GdnHCl on the strain-specific PrP conversion reactions. The final GdnHCl concentration in the reaction was adjusted to either 1, 2, or 3 M. The reactions were diluted to 0.4 M GdnHCl just prior to digestion with PK. ^{35}S-PrP^C in the absence (-) of PrP^{Sc} was not treated with PK. The arrowhead marks the molecular weight of the 24 kDa ^{35}S-PrP^C precursor and the molecular weight of the marker proteins is indicated at the left. The absence of PK resistant products after incubation in 3 M GdnHCl was likely due to irreversible denaturation of PrP^{Sc} rather than ^{35}S-PrP^C; pretreatment of ^{35}S-PrP^C with 7.5 M GdnHCl did not inhibit its conversion in earlier studies.

Figure 5.3 The effects of detergents on the conversion of ^{35}S-PrP^C to the protease-resistant form. The first lane on the left shows the product of a conversion reaction as described in the legend of Fig. 5.1 in the absence of detergent. The next lanes were conversions done identically except for the presence of the indicated amount of CPC or Triton X-100 in the reaction mixture. All reactions contained 80,000 cpm of ^{35}S-PrP^C initially. Molecular weight markers are indicated at the left in kilodaltons.

Figure 5.4 The effects of CPC on the strain specific PrP conversion reactions. The conversion reaction was performed in the presence (+) or absence (-) of 1.5 mM CPC. Molecular weight markers, in kilodaltons, are indicated at the right. In the presence of CPC, the major PK resistant ^{35}S-PrP conversion products had molecular weights of 18 to 22 kDa after incubation with HY PrP^{Sc}. In the DY PrP^{Sc} reaction, the addition of CPC did not change the migration of the major 16

kDa conversion product although different minor conversion products were formed.

Figure 5.5 Effect of PK pretreatment of PrP^{Sc} on the strain-specific cell-free conversion reaction. HY and DY PrP^{Sc} were digested (+) with PK (pre) to generate preparations highly enriched for PrP^{Sc} (>95% by SDS-PAGE). Predigested and undigested (-) PrP^{Sc} were used in the cell-free conversion assay followed by the PK digestion step (post), as described in Fig. 5.4. The arrowhead marks the 24 kDa ^{35}S-PrP^{C} GPI^{-} precursor.

Figure 5.6 The conversion of unglycosylated ^{35}S-PrP^{C} to PK resistant forms. Cells treated with or without 15 μg/mL tunicamycin were immunoprecipitated and the untreated labels are shown in lanes 1 and 2. The arrow points to the monoglycosylated 28 kDa form and the arrowhead points to the unglycosylated 24 kDa form. Conversion reactions were done with glycosylated and unglycosylated ^{35}S-PrP^{C} with HY (H) and DY (D) PrP^{Sc}. Note that the banding patterns are identical. Molecular weight markers are indicated at the right in kilodaltons.

Figure 5.7 Nucleation-dependent protein polymerization model for strain differences in scrapie and other TSEs. Monomeric PrP^{C} is soluble and kinetically stable. In the presence of a PrP^{Sc} nucleus or seed, which is an ordered polymer, growth by addition of a PrP monomer (indicated by shaded rectangles) is favored. The monomeric PrP^{C} or, perhaps, an unfolded intermediate (PrP^{u}) acquired a new conformation that is stabilized by intermolecular interactions within the PrP^{Sc} polymer. In this model, strain differences would be due to PrP^{Sc} polymers that have distinct packing arrangements and/or conformations. The same monomeric PrP conformer could be packed into differently arranged PrP^{Sc} polymers (as illustrated in the figure) or distinct PrP conformers could be incorporated into different PrP^{Sc} polymers. PK digestion of different PrP^{Sc} polymers could generate distinct cleavage products even if they are derived from the same PrP^{C} precursor.

Fig.5.1

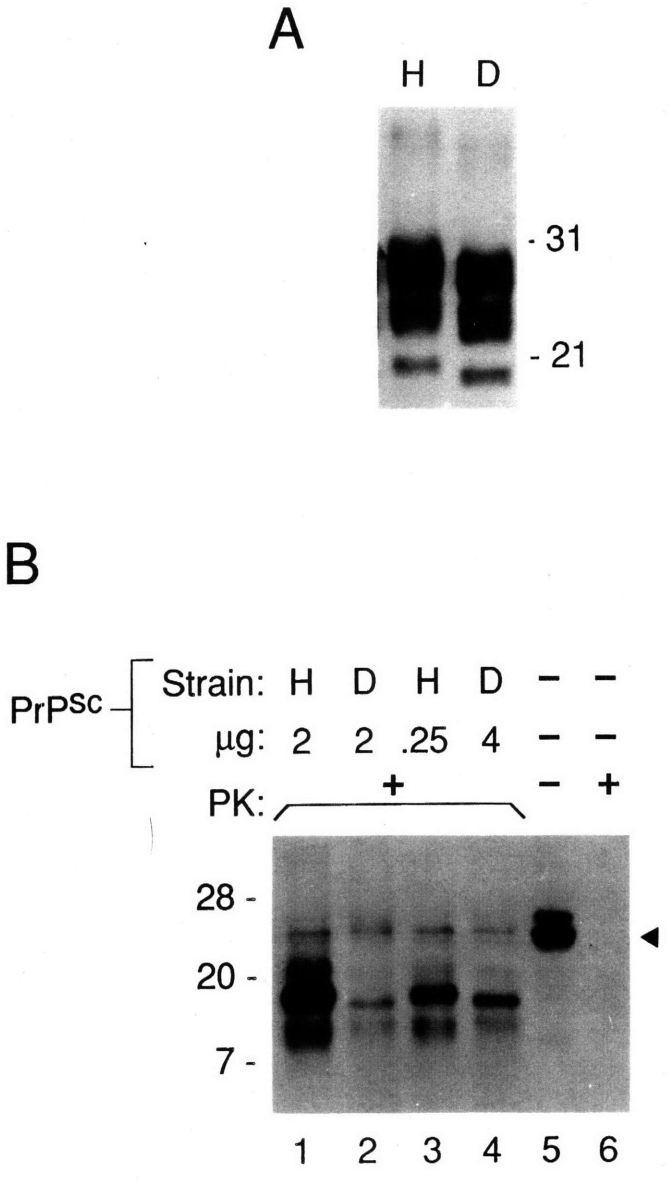


Fig.5.1C

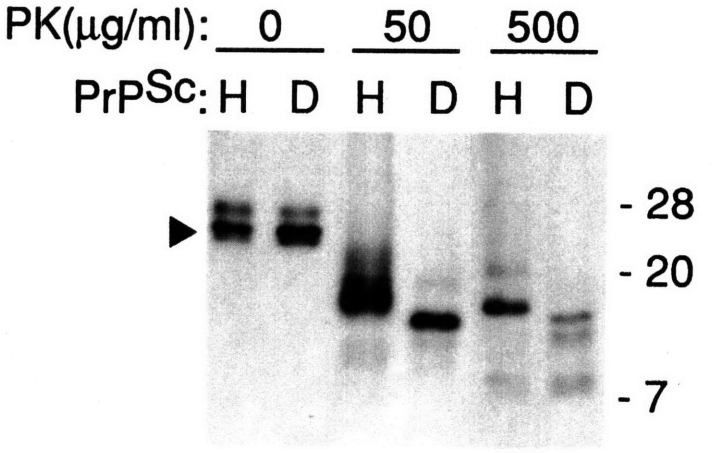
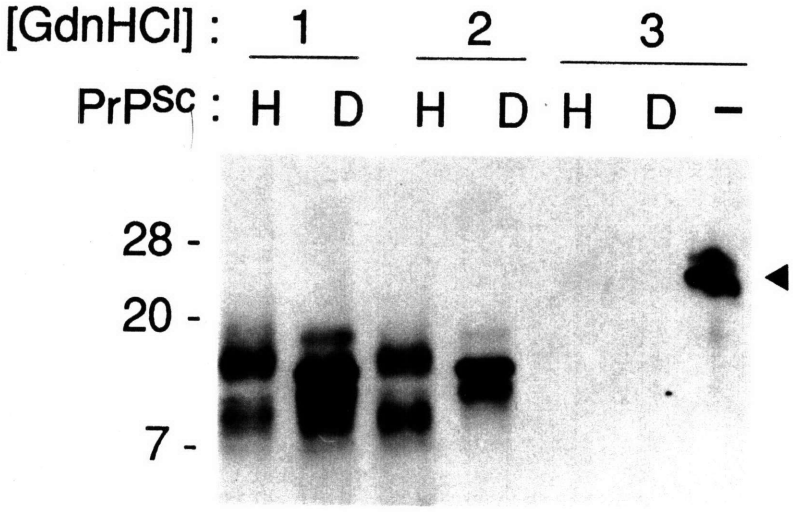


Fig.5.2



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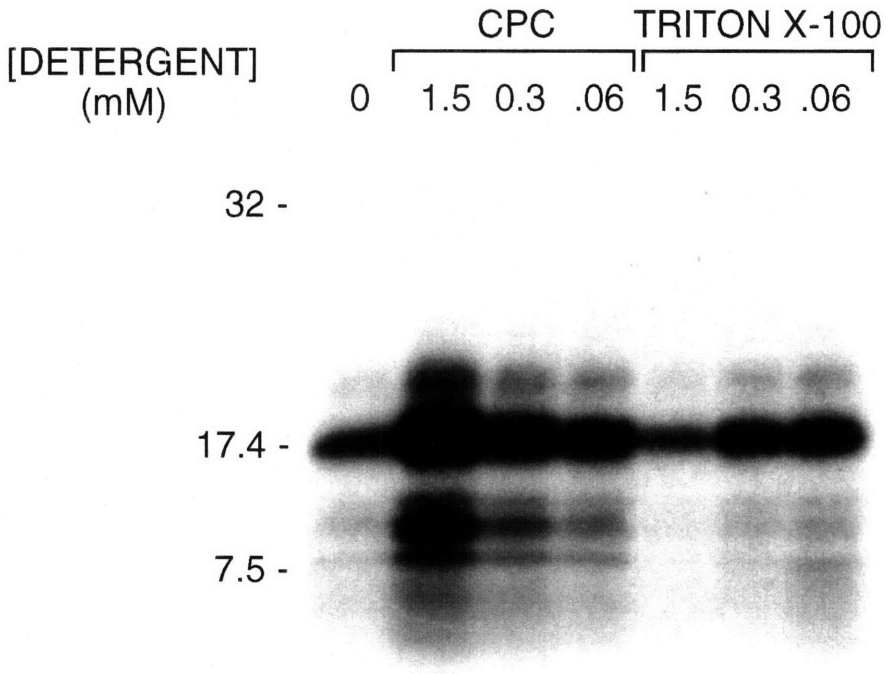

Fig.5.4

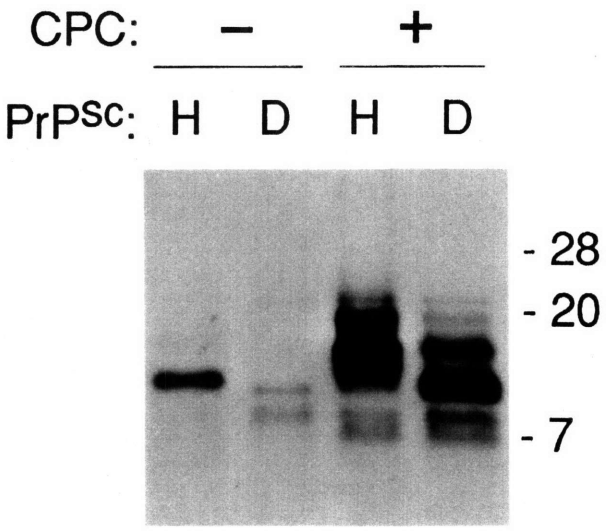

Fig.5.5

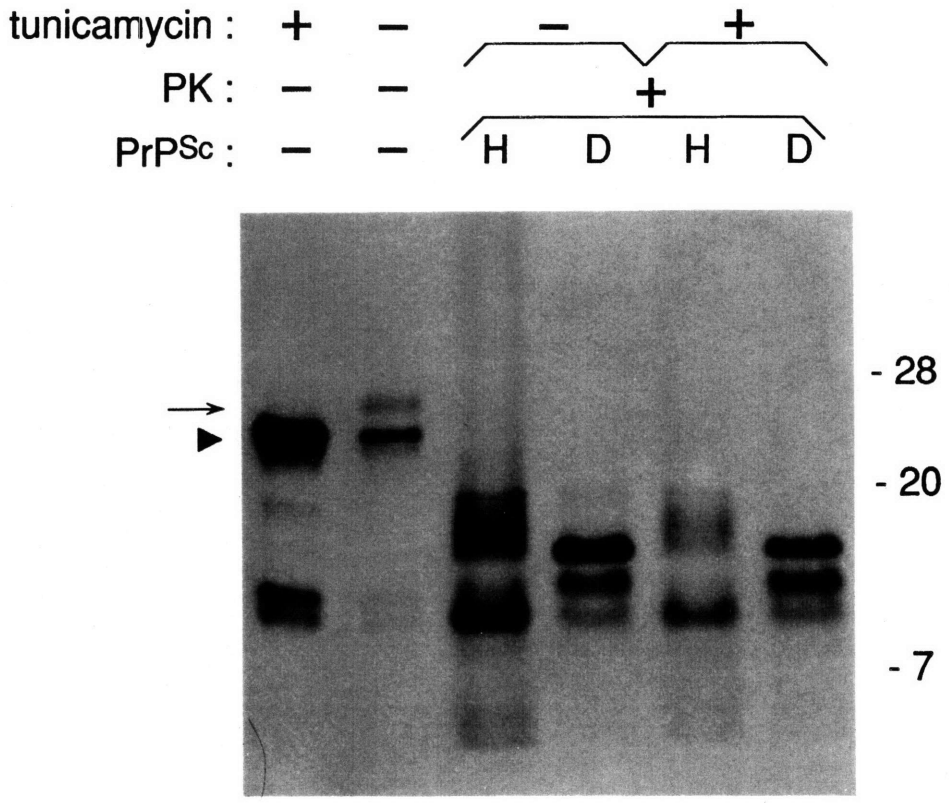

Fig.5.6