CONFORMATION BASED REAGENTS FOR THE DETECTION OF DISEASE-ASSOCIATED PRION PROTEIN

By

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To Grandad Dee, for teaching me to love science,
and Grandad Hatcher, for always believing I could do it

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LIST OF ABBREVIATIONS

BSE	Bovine spongiform encephalopathy
CJD	Creutzfeldt-Jakob Disease
fCJD	Familial Creutzfeldt-Jakob disease
iCJD	Iatrogenic Creutzfeldt-Jakob Disease
sCJD	Sporadic Creutzfeldt-Jakob Disease
vCJD	Variant Creutzfeldt-Jakob Disease
CSF	Cerebrospinal Fluid
C-Type BSE	"Classical" BSE
CWD	Chronic Wasting Disease
DELFIA	Dissociation-enhanced lanthanide fluorescence immunoassay
EEG	Electroencephalogram
ELISA	Enzyme-Linked Immunosorbent Assay
EMSA	Electrophoretic Mobility Shift Assay
FFI	Fatal Familial Insomnia
GSS	Gerstmann-Straussler-Scheinker disease
H-Type BSE	BSE in which unglycosylated band migrates higher than C-type
K(1+2+3)	The first three kringle domains of plasminogen (also called angiostatin)
NMR	Nuclear Magnetic Resonance
PSWC	Periodic Sharp Wave Complex
PrP	Prion protein
PrP^{C}	Cellular prion protein
PrP^{Sc}	Disease-associated (scrapie) prion protein
PK	Proteinase K
rhu PrP^{C}	Recombinant human cellular prion protein
SELEX	Systematic evolution of ligands by exponential enrichment

Conformation Based Reagents for the Detection of Disease-Associated Prion Protein

ABSTRACT

by

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Prion diseases are a group of fatal, transmissible neurodegenerative diseases found in both animals and humans. Prion diseases are thought to arise when the normal cellular prion protein, PrP^{C}, undergoes a conformational change from a primarily α-helical form into a form rich in β-sheets and resistant to protease, called PrP^{Sc}. Animal forms of prion disease include scrapie in sheep, chronic wasting disease (CWD) in cervids, and bovine spongiform encephalopathy (BSE) in cattle. In humans, the diseases can take a sporadic, genetic or acquired form. One acquired form of human prion disease is variant Creutzfeldt-Jakob disease (vCJD), which emerged in the United Kingdom in 1995, and is thought to be caused by the consumption of BSE contaminated beef. The only definitive diagnostic techniques available at the present time use protease digested brain tissue.

This thesis sought to develop a panel of diagnostic reagents for prion disease that enable the detection of PrP^{Sc}, but not PrP^{C}, without protease digestion. We characterized two short peptide sequences, based on the Kringle domains of the serine protease plasminogen, for binding to PrP^{Sc}, following reports that plasminogen has PrP^{Sc} binding ability. The short peptides bound to all forms of PrP^{Sc}, both animal and human, and binding was retained in PrP^{Sc}-spiked human plasma. In addition, a panel of PrP binding DNA oligonucleotides, called aptamers, isolated through the SELEX technique, was used

in a capture assay. The aptamers bound to full length and PK treated PrP^{Sc} from hamster scrapie, as well as from sporadic CJD, vCJD, mouse scrapie, sheep scrapie, and white-tailed deer derived CWD. Binding was not observed to PrP^{Sc} from mule deer CWD, or from BSE, making these reagents among the first to show species or strain specificity. Strikingly, these aptamers were able to distinguish buffy coat samples derived from scrapie afflicted sheep from those of healthy animals in an electrophoretic mobility shift assay with 96% sensitivity and 100% specificity. These peptides and aptamers represent novel reagents with potential to open new avenues in prion disease research, surveillance, and therapeutics.

CHAPTER 1

INTRODUCTION

1.1 The Prion Protein

The prion is the pathologic agent responsible for the transmission and propagation of prion diseases (1, 2). Prion diseases are a group of fatal, transmissible neurodegenerative disease, also known as transmissible spongiform encephalopathies (TSEs) that occur in both humans and animals(3). Prion diseases arise as result of one of three etiologies; sporadic, genetic or infectious. Animal prion diseases include scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in cervids, as well as several rare forms of the disease arising in felines, mink and exotic ungulates. In humans, prion diseases most often occur in the form of sporadic Creutzfeldt-Jakob disease (sCJD), with an incidence of 1 case per million population annually (4). Creutzfeldt-Jakob disease may also arise as a result of a mutation in the gene encoding the prion protein (*PRNP*) in the form of familial Creutzfeldt-Jakob disease (fCJD)(5). Additional forms of genetic prion disease include fatal familial insomnia (FFI) and Gerstmann-Sträussler-Scheinker disease (GSS)(5). Finally, prion diseases in humans may occur as a result of infection, as in the case of iatrogenic Creutzfeldt-Jakob disease, which occurs during the course of certain medical procedures, and variant Creutzfeldt-Jakob disease (vCJD), which is believed to occur as a result of consumption of BSE infected tissue (6-10).

Prion diseases are thought to arise as a result of a conformational change occurring in the normal cellular prion protein, PrP^{C}(11). PrP^{C} is a conserved, glycophosphatidylinositol anchored protein found to be highly expressed in the nervous

system. Structurally, PrP^C contains three alpha helices and two beta sheets in the C terminal region. The N terminal region is poorly defined structurally, but contains several octapeptide repeats (sequence: PHGGGWGQ) which are known to bind copper *in vivo*(12). In addition, there are two non-obligatory sites for N-linked glycosylation. In the cell, PrP^C is generally localized in cholesterol-rich regions of the membrane called calveolae, or lipid rafts, although PrP^C localized to the cytosol has also been discovered(13). Despite its expression in the brain and numerous peripheral tissues such as lung, heart, kidney, muscle and gastrointestinal tract, as well as in lymphoid cells, the physiological role of PrP^C is not well understood(14). However, it has been implicated in processes as diverse as neurotransmitter metabolism, apoptosis, copper metabolism, neuroprotection, signal transduction, and immune cell activation(14).

Mice lacking expression of the prion protein were first generated in 1993(15). Although these animals were found to be resistant to experimental infection with scrapie, there were no major developmental deficits(15). This is one of the main reasons that the biological function of PrP^C has remained elusive. More recent studies on knockout mice have described subtle phenotypic variations, including disruptions in sleep and circadian rhythms, synaptic transmission and morphologic alterations in the hippocampus(14). In addition, age-dependent memory impairment was observed in certain strains of PrP -/- mice, suggesting that PrP^C loss of function has cognitive implications. The variety of phenotypes reported in PrP -/- mice suggest that PrP^C regulates a wide array of functions, or that it has a role early in development, from which diverse consequences arise(13).

The biological function of PrP^C has important implications for understanding the pathogenesis of prion diseases. The major site of prion disease pathology is the central

nervous system, where the prion protein is most highly expressed. It is currently not clear whether the pathological changes that occur during the course of prion diseases are a result of loss of function of PrP^{C} or gain of function of PrP^{Sc}. While some authors suggest that the neuropathology of prion disease is explained by loss of function, others suggest that the minor phenotypic changes in PrP -/- mice indicate that loss of PrP^{C} has no serious consequences, therefore a pathogenic gain of function upon conversion to PrP^{Sc} is more likely(13). However, if PrP^{C} does indeed play a protective role in the neuron, as suggested by potential antioxidant activity and modulation of apoptosis, the loss of this activity upon conversion to PrP^{Sc} could prove to be neurotoxic(13).

1.1.1 The Codon 129 Polymorphism

At codon 129 of the prion gene, either a methionine or a valine residue is encoded. This polymorphism is a critically important determinant of susceptibility, age of onset, and disease duration in the prion diseases(16). For example, methionine homozygosity at codon 129 has been exclusively linked to the development of variant Creutzfeldt-Jakob disease following exposure to bovine spongiform encephalopathy(6). In addition, homozygosity is linked to an increased risk of developing sporadic or iatrogenic Creutzfeldt-Jakob disease(16). Perhaps the most striking example of the modulating effect of the codon 129 polymorphism on disease phenotype is that observed with the D178N mutation. When this mutation occurs in conjunction with methionine at position 129, the disease phenotype is fatal familial insomnia. If valine is at position 129 on the mutant allele, the result is familial Creutzfeldt-Jakob disease(5).

Despite the obvious importance of this polymorphism to the susceptibility, progression and phenotype of prion disease, the mechanism underlying these effects is

not well understood. Methionine and valine are relatively similar in terms of volume and hydrophobicity, and NMR studies reveal no obvious structural differences between the two variants(17). However, recent studies suggest that PrP^{C} containing methionine forms amyloid fibrils more readily than the valine variant, perhaps due to exposure of helix 1, a region believed to be critical in the conversion of PrP^{C} to PrP^{Sc}. Helix 1 contains six charged residues oriented towards the surface of PrP^{C}, and these are thought to form intermolecular ionic bonds with the same location on other PrP^{C} molecules, therefore promoting aggregation(18, 19). However, residue 129 is not itself located in helix 1, so the structural basis of its potential effect on the position of helix 1 is unclear. However, it has been demonstrated that the methionine variant recruits other PrP molecules into aggregates more efficiently, providing a potential biochemical basis for its effect on disease susceptibility(18, 20).

1.2 The Pathogenic Prion Protein

1.2.1 Prion Conversion

The mechanistic details of the pathogenesis of prion diseases are poorly understood. The prevailing hypothesis, known as the protein only hypothesis, holds that PrP^{C} undergoes a change in conformation to the pathogenic PrP^{Sc} form(2). Although the structure of PrP^{Sc} has not been solved, it has been determined that it is rich in beta sheets. Fourier-transform infrared spectroscopy and circular dichroism studies indicate that while PrP^{C} contains approximately 40% alpha helix, and only a small amount of beta sheet, PrP^{Sc} contains 45% beta sheet, and about 30% alpha helix(11). It differs from PrP^{C} in that it is insoluble in nonionic detergents, has a propensity for aggregation, and is partially resistant to protease digestion. This latter property is the biochemical hallmark

of PrP^{Sc}, and is an important characteristic that enables differentiation of the two prion protein conformations.

Two different models have been proposed for the conversion of PrP^{C} into PrP^{Sc}. The first is the nucleated polymerization model, proposed by Jarrett and Lansbury in 1993(21). This model hypothesizes that PrP^{C} can spontaneously convert into PrP^{Sc}, but this process is inhibited by a significant energy barrier. When a critical quantity of misfolded protein accumulates, or when a significant quantity is introduced exogenously, small PrP^{Sc} aggregates form(21). These initial aggregates act as a seed, recruiting further PrP molecules to form larger aggregates. The incorporated PrP monomers are rapidly stabilized by conversion into PrP^{Sc}, and as larger multimers form, fragmentation can then produce more seeds, and PrP^{C} conversion proceeds rapidly.

The second conversion model, proposed by Prusiner and colleagues, relies on template-directed misfolding(22). Again, upon random misfolding of PrP^{C}, or through introduction of exogenous material, PrP^{C} is recruited for conversion. However, this model proposes that monomeric PrP^{Sc}, through dimerization with individual PrP^{C} molecules, is responsible for recruitment and conversion. Aggregates form following this process, and are not responsible for the conversion itself(22).

The nucleation-seeding conversion model is supported by work from Silveira et al., in which PrP^{Sc} containing aggregates were partially disaggregated and fractionated by size(23). Bioassays and in vitro conversion assays revealed that the highest infectivity was associated with non-fibrillar particles of 300-600 kDa, which is equivalent to 14-28 PrP molecules. Furthermore, virtually no infectivity was associated with oligomers of less than five PrP molecules(23). With the absence of infectivity in PrP monomers, it

seems unlikely that monomeric PrP^{Sc} would be responsible for propagating prion disease through the recruitment and conversion of individual PrP^{C} monomers. Rather, it appears that the small aggregates could instead act as seeds, as proposed in the nucleation-seeding model. Therefore, of the competing models, current evidence favors the nucleation-seeding model.

1.2.2 Proteinase K Sensitive PrP^{Sc}

The existence of Proteinase K (PK) sensitive PrP^{Sc} was first described by Safar et al, in the conformation-dependent immunoassay(24). In PK untreated samples, immunoreactivity of PrP^{Sc} was enhanced as partial denaturation uncovered buried epitopes. This effect was lost in PK treated samples, indicating that there is a subset of PrP^{Sc} that is PK sensitive. When subjected to sucrose gradient fractionation, it was discovered that PrP from infected brains distributed in a continuum of aggregates, with the larger, denser fragments resistant to PK digestion(25). There were smaller oligomers recovered from intermediate fractions that were PK sensitive, and these did not appear at all in gradients of control brains. This suggests that PrP^{Sc} is in fact a heterogeneous collection of oligomers, and that its hallmark protease resistance is dependent, at least in part, on quaternary structure. Studies using the conformation-dependent immunoassay to probe the characteristics of PrP^{Sc} in the brains of CJD patients revealed that as much as 80% of the PrP^{Sc} present is PK sensitive(26). As PK digestion is the method currently relied upon for the identification of CJD, it would appear that the amount of PrP^{Sc} present in these samples is largely underestimated. This may be of particular importance if the majority of PrP^{Sc} present in bodily fluids, such as blood or urine, is also PK sensitive. The diagnostic test with the best chance of detecting the small amounts of PrP^{Sc} expected

to be present in these fluids, then, is one that relies on reagents with the ability to differentiate PK sensitive PrP^{Sc} from PrP^{C}.

1.2.3 Prion Strains

Despite the absence of nucleic acid in the prion agent, a species barrier persists in the transmission of prion diseases. Prion diseases occur in some animals, but not others, and in infection between closely related species, variations in the rate of infection and incubation period are seen as species barrier effects(27). Typically, the initial infection of a particular species with infectious material from another species is inefficient, with a prolonged incubation time, if clinical disease appears at all(28). Further passages usually result in shorter incubation periods, presumably the result of adaptation of the PrP^{Sc} to the new host, and stabilization of the strain(29). This species barrier is thought to be responsible for the rarity of natural infection observed between species, for example the lack of human disease associated with the widespread consumption of lamb and mutton in scrapie endemic areas. However, there have been instances of cross-species infection in nature, most prominently, the occurrence of vCJD(30, 31). In addition, meat and bone meal in the pet food industry has lead to the BSE infection of domestic cats, in the form of feline spongiform encephalopathy(32).

The species barrier in prion diseases is currently attributed to differences in the primary sequences of PrP, as well as subtle differences in the folding of PrP^{Sc}. The differences in folding of PrP^{Sc} are thought to result in differential cleavage by PK, and variations of unfolding rates in denaturation assays(24, 33). In the former case, the tertiary conformation of PrP^{Sc} dictates which sites are accessible to PK for cleavage, with conformation variations resulting in fragments of different sizes. In the latter case, highly

sensitive assays such as the conformation dependent immunoassay, discussed later, are used to detect the differences in rates of unfolding(24).

The conformational differences in prion strains were demonstrated in work by Safar et al., in which it was demonstrated through the development of the highly sensitive conformation-dependent immunoassay (CDI)(24). Using a fluorescence-based detection system, eight different strains propagated in Syrian hamsters were tested. Clear differences were seen in the unfolding rates when the PrP was exposed to increasing concentrations of guanidine HCl. This assay proved to be more effective at differentiating strains than PK digestion. Seven of the eight strains tested were indistinguishable after PK digestion by Western blot, yet all strains had unique unfolding profiles under the conditions of the CDI experiment. Not only do these findings impact the understanding of prion diseases, but they also challenge one of the fundamental tenets of protein biochemistry, that a primary sequence determines a single structure(24).

1.3 Creutzfeldt-Jakob Disease

1.3.1 Sporadic CJD

Sporadic CJD typically affects individuals in the sixth to seventh decade of life, with an incidence of approximately one case per million persons per year(34). There is no evidence of geographical clustering. Recent studies have suggested links with occupation and previous surgery as increasing the risk for later development of sCJD. Occupations associated with increased risk include butchers and individuals working in the health care field, such as those working in physician offices. While no significant correlation was observed with the development of CJD and neurological surgery specifically, it was demonstrated that increase in risk was strongest in three categories:

skin stitches, nose/throat surgery and removal of cysts and moles in the 10-20 years preceding the onset of sCJD symptoms(35). However, it is not clear if such an association is truly causal, or suffers from recall bias.

The classic symptoms of typical sporadic Creutzfeldt-Jakob disease, identified as subtype MM/MV1, are rapidly progressive dementia, myoclonus, and in some cases, ataxia(34). The phenotypes for other subtypes of sCJD vary. At disease onset, there are often no specific symptoms, and in a small percentage of cases, patients may exhibit psychiatric symptoms, including hallucinations and delusions. As the disease progresses, multi-focal central nervous system failure occurs, resulting in rigidity, cortical blindness, dysphagia and possibly Cheyne-Stokes respiration at the terminal stage(34). The mean duration of the disease is approximately 8 months, with less than 4% of patients surviving longer than two years(34). The most important differential diagnoses include Alzheimer's disease, vascular dementia, diffuse Lewy body disease, brain tumors and cerebellar degeneration(34).

As a result of the wide spectrum of clinical signs and pathological changes observed in sCJD, attempts have been made to classify the disease into subtypes. The most widely used classification system segregates the disease according to zygosity at codon 129, and the presence of one of two biochemically distinct types of PrP^{Sc}(36). The two types of PrP^{Sc} are distinguished by the migration of the unglycosylated band following PK digestion. If the band migrates to 21 kDa, this represents type 1 PrP^{Sc}, while migration to 19 kDa represents type 2 PrP^{Sc}. These migration patterns are a result of differential cleavage of PK, possibly due to conformational differences in the two types(37). The dominant N terminus of type 1 occurs at residue 82, while the N terminus

of type 2 is located at residue 97. These two types are thought to represent two different prion strains(37). Under this classification system, classic sCJD, the most common type accounting for approximately 70% of cases is designated MM1. There are 6 subtypes in total, corresponding to the presence of type 1 and type 2 PrP^{Sc} in each of the three possible codon 129 genotypes(36). It has been proposed that the phenotype of sCJD is determined by the interaction between the type of PrP^{Sc} and the codon 129 genotype. The variations in clinical presentation are summarized in Table 1.

Table 1. Molecular subtypes of sCJD with corresponding phenotypic features(38)

sCJD type	Variant	% of cases (this study)	Key features
MM1, MV1	Classic, typical	56	Rapid progression, short duration, periodic sharp waves on EEG, prominent and early myoclonus
VV2	Ataxic	21	Ataxia at onset, dementia late, no periodic sharp waves on EEG
MM2	Slowly progressive	11	Progressive dementia, no periodic sharp waves on EEG, large vacuoles on histology
MV2	Ataxic with Kuru plaques	8	Ataxia and dementia, no periodic sharp waves on EEG, some with long duration, Kuru plaques in cerebellum
VV1	Early onset	3	Progressive dementia, no periodic sharp waves, younger patients

A second classification system was proposed by Collinge and colleagues, recognizing three prion subtypes in sCJD, and a separate subtype for vCJD(39, 40). The main difference between the two classifications is that type 1 in the Collinge classification appears to migrate approximately 0.5 kDa higher than type 1 of the previous classification, while Collinge types 2 and 3 correspond to types 1 and 2 described above. Attempts to reconcile these classification systems have been largely unsuccessful(41, 42). The Collinge classification essentially subdivides the MM1 subtype of CJD into two distinct phenotypic groups, which differ on the basis of disease duration. It appears, however, that the small migration difference between Collinge type 1 and type 2 may be due to varying experimental conditions, and lacks distinct histopathological characteristics(41). This lack of clarity in the classification of sCJD may impede the characterization of CJD subtypes, including those that may be infectious in origin.

1.3.2 Familial CJD

All known incidents of familial Creutzfeldt-Jakob have been caused by mutations in *PRNP*(43). Familial CJD is unusual among genetic neurological disorders because of the heterogeneity in disease phenotype, despite all known mutations originating within a single gene. In total, fCJD accounts for approximately 10-15% of all CJD cases. There are over 30 different mutations described in the literature, and these fall into two different categories: the first are point mutations leading to amino acid substitutions or premature stop codons, and the second are insertions of additional octapeptide repeats(5).

Mutations in PRNP are inherited in an autosomal dominant manner. Historically, genetic prion diseases have been divided into three distinct diseases: Gerstmann-Sträussler-Scheinker disease (GSS), fatal familial insomnia (FFI) and familial Creutzfeldt-Jakob disease (fCJD). The core features of these diseases are slow progression of ataxia and late onset dementia in GSS; insomnia, hallucinations, dysautonomia and motor signs in FFI; and rapidly progressive dementia with myoclonus most commonly in fCJD(43).

The most common mutations causing fCJD are E200K and D178N/129V. The clinical and histological features of E200K are similar to those of classical sCJD (MM1/MV1). All patients eventually develop dementia and other cognitive and psychiatric disturbances, and a majority of patients develop myoclonus and seizures. The frequent involvement of the peripheral nervous system in this form of fCJD serves to differentiate it from sCJD, where this symptom is rarely observed(44, 45). The largest cluster of E200K occurs among Jews of Libyan descent, with other clusters throughout the world(46). The age at disease onset is variable, ranging from the fourth to the eighth decades of life, with the mean age of onset at approximately 60 years. By the ninth decade, life table analysis reveals a near-complete penetrance of the disease(47).

The first case of familial CJD recorded in 1924 was a member of a German kindred, eventually determined to be a result of the D178N/129V mutation(5). The disease duration and age at onset in this type of fCJD is determined by the zygosity at codon 129(48). The average age at disease onset is 39 years in patients homozygous for valine, and 49 years in heterozygous patients. Heterozygous patients have a longer disease course, averaging 27 months, compared to 14 months in homozygous patients. The clinical signs are consistent regardless of zygosity, and include cognitive

impairment, depression, irritability and abnormal behavior. Ataxia, speech impairments and myoclonus frequently develop during the course of the disease(49). There are several less common mutations associated with a CJD phenotype, which affect a small number of patients. Some of these have been found in single individuals without family history. Because linkage analysis for many of these mutations is unavailable, it is not certain that the mutations are causally linked to the disease(5).

In certain cases, fCJD is not caused by amino acid substitution in PrP^C, but instead by insertion of octapeptide repeats in the N-terminal region of the protein. Normally, PrP^C contains five octapeptide repeats, and insertion of one to nine extra repeats has been associated with fCJD. The first insertion mutation case was of six octapeptide repeats in a British family, but other families have since been found around the world(5, 50). In the majority of families, the insertion mutations are associated with methionine at codon 129. The phenotype of insertion mutations is highly variable, appearing to be dependent on the number of repeats. The phenotype of patients with four or fewer repeat inserts is almost identical to sCJD. Patients with five to seven repeats are also phenotypically similar to sCJD, but with an earlier age of onset and a slower disease progression, and those with eight or nine inserts display a GSS-like phenotype with widespread deposition of PrP amyloid plaques. Deletion of repeats has also been described. While the deletion of one repeat is a common polymorphism affecting 1-2% of the general population, deletion of two repeats is associated with disease(5).

Fatal familial insomnia (FFI) is the most dramatic example of phenotypic heterogeneity among familial prion diseases. Although it is caused by the D178N mutation, which is associated with the CJD phenotype described above, it has a unique

phenotype resulting from the codon 129 polymorphism in the affected allele(48). Whereas the CJD phenotype is associated with D178N/129V, the FFI phenotype is associated with D178N/129M. In addition to determining the disease phenotype, the zygosity at codon 129 modulates disease severity and duration. Methionine homozygotes have disease duration of approximately 12 months, while the duration in heterozygotes is approximately 21 months(51). Clinically, FFI involves abnormalities in three physiological activities: sleep, and autonomic and motor functions(5).

1.3.3 Acquired CJD

1.3.3.1 Iatrogenic CJD and Kuru

Iatrogenic CJD arises through the transmission of prions as a result of a medical procedure. The most common mechanisms of disease transmission occur as a result of dura mater grafts, cadaver-derived growth hormone, and contamination of neurosurgical instruments(8-10, 52). The contamination of all these cases likely arises from sCJD, so due to the rarity of that disease, iatrogenic CJD is correspondingly rare. There are only a handful of cases reported to the National CJD Surveillance Unit in the UK each year, with some years reporting no cases at all. Iatrogenic CJD occurring as a result of surgery occurs because PrP^{Sc} is resistant to the standard protocols for surgical instrument sterilization. Having recognized this problem, there has been much work done to develop effective sterilization techniques to destroy prions in both neurosurgical and dental instruments(53, 54). However, the discovery of PrP^{Sc} accumulation in non-neurological tissue, such as the spleen and tonsils, raises new questions about the safety of surgical instruments outside of neurosurgical procedures. Therefore, reagents to ensure the sterility of surgical instruments are of paramount importance.

Kuru is a prion disease affecting the Fore linguistic group in the Eastern highlands of Papua New Guinea(55). It occurred as a result of the practice of mortuary feasting, in which deceased relatives were consumed as a mark of respect and mourning. Kuru deaths were recorded continuously from 1957. Accounts from oral historians place the first remembered kuru case in the early 20th century. The incidence rapidly increased, reaching a peak annual mortality of 2% in the worst affected villages. The victims appeared to be largely adult women and children, consistent with cultural practice rather than a particular susceptibility in these groups. The initial case is thought to have occurred as a chance consumption of an individual who perhaps died from sCJD(55). Kuru was also the first human prion disease proven to be transmissible, with the inoculation of nonhuman primates with autopsy-derived brain tissue. Kuru is valuable as a model of human prion disease transmission, especially in light of the emergence of vCJD. The practice of mortuary feasting among the Fore ceased in the late 1950s, yet cases have continued to emerge in the last decade. This indicates an incubation period of 50 years or greater(55). Interestingly, the longer incubation periods of kuru are associated with heterozygosity at codon 129, while susceptibility to kuru is increased in methionine homozygotes(55).

1.3.3.2 Variant CJD

Perhaps the biggest threat to public health of prion diseases is the emergence of variant CJD. This disease first occurred in the United Kingdom in 1995, shortly following the peak of BSE incidence(6). The correlation of BSE and vCJD, both temporally and geographically suggests a causal link between the two diseases. This association has been further explored with molecular strain typing of the prions from both

diseases, and through transmission studies to mice(30, 31). The PrP^{Sc} from BSE and vCJD show similar migration patterns and glycoform ratios, and BSE is transmissible to transgenic mice over expressing the human prion protein. There are, however, significant differences in the pathogenesis and brain pathology of the two diseases. BSE in cattle does not show significant involvement of the lymphoreticular system, whereas the lymphoreticular involvement in vCJD is strong. PrP^{Sc} has been found to accumulate in the germinal follicles of lymph nodes, particularly within gut-associated lymphoid tissue, and the spleen(56). In vCJD, morphological changes are seen only in the central nervous system, despite the extensive accumulation of PrP^{Sc} within the lymphoreticular system. This extensive lymphoreticular PrP^{Sc} accumulation is unique to vCJD among human prion diseases. Pathologically, spongiform change in the brain is a characteristic feature, as is the case with other prion diseases(56). In vCJD, the caudate nucleus, putamen and cerebellum show particularly prominent spongiform change. In addition, the pathology of vCJD includes astrocytosis and neuronal loss, particularly severe in the pulvinar(56). This serves as the basis for the so-called "pulvinar sign", a characteristic high signal observed in MRI images of vCJD brains(57). PrP^{Sc} accumulates in the form of florid plaques, which are plaque-like depositions of PrP^{Sc} surrounded by a halo of spongiform change(34).

With growing evidence for a causal link between the BSE epidemic and vCJD in the UK, the widespread exposure of the UK population to BSE contaminated food products is of great concern. It is estimated that over 400 000 infected cattle may have entered the human food chain prior to the meat and bone meal ban(56). Fortunately, the vCJD incidence to date has remained lower than many predictions; however, it is too

soon to say that the epidemic has passed. Two large scale screening studies, in which tonsillectomy and appendectomy specimens from the general population were tested for PrP^{Sc} accumulation, have been completed(58). In the first study, no positive cases were found using either immunohistochemistry or Western blot. However, in this study, the median age of patients was under 10, a group that would not have had significant BSE exposure(59). A second study concentrated on the 10-30 age group, at which vCJD reaches peak incidence. Three positives were found in this study, giving an approximate prevalence of 237 cases per million persons(58). Although vCJD cases over the past several years have been steadily declining, this fails to take into account the prevalence of asymptomatic carriers, cases that may arise through secondary transmission, such as blood transfusion, and possible longer incubation periods among those who are not methionine homozygotes(60, 61).

The transmission of kuru, and to a lesser extent, iCJD, can potentially serve as a model for the vCJD epidemic. In both cases, methionine homozygotes were most susceptible, as has so far been the case with vCJD(55). The median incubation time for kuru is estimated to be 10-13 years, and for iCJD following injection of cadaver-derived human growth hormone, 12-17 years(55). However, in certain cases, the incubation times of both diseases have been as long as 40 years or more. As mentioned previously, longer incubation times are associated with heterozygosity or valine homozygosity at codon 129. There is no reason not to assume the same may be true for vCJD, especially in light of the discovery of PrP^{Sc} accumulation in the lymphoreticular tissue of the heterozygotic individual. Therefore, based on these findings, vCJD is predicted to appear

in other codon 129 groups. It may also be possible that other genes may play a role in determining susceptibility and incubation times(62).

Studies in transgenic mice have been performed to examine the transmission vCJD to different codon 129 genotypes(63). The transgenic mouse models show that infection of heterozygotes or valine homozygotes does occur with inoculation of vCJD or BSE prions, but that this may result in the propagation of different prion strain types. The rate of infection was not 100% in either the MV or the VV transgenic groups, but in those mice that were affected, the clinical, pathological and molecular phenotypes were distinct from methionine homozygote infection(63). No confirmed case of vCJD has occurred in MV or VV human patients, but in 2007 a report emerged of a young female patient, a valine homozygote, presenting with a prion disease phenotypically distinct from sCJD(64). Molecular analysis of the PrP^{Sc} revealed a glycoform ratio and fragment size resembling that seen in vCJD under standard conditions, but differed from vCJD upon addition of the metal ion chelator EDTA. Variation in PK cleavage sites with EDTA addition is seen in PrP^{Sc} associated with some types of sCJD, but has not been documented with vCJD prions. However, the molecular characteristics of the PrP^{Sc} from this patient shared similarities with VV transgenic mice that had been inoculated with vCJD(64). Although this case was not classified as vCJD, it raised questions about two other cases of prion disease in valine homozygotes. In both cases, the initial classification was sporadic, despite unusually young patients and an atypical disease phenotype. While the connection between these cases and vCJD or BSE exposure has not been conclusively demonstrated, it clearly illustrates the importance of careful examination and continued surveillance of human prion diseases.

1.4 Chronic Wasting Disease (CWD) and Bovine Spongiform Encephalopathy

Although BSE has not been a serious problem in North America, CWD is endemic in several western states in the United States, and in two Canadian provinces. In Colorado and Wyoming, for example, as much as 15% of the free-ranging mule deer and elk population are infected(65). The mode of transmission of CWD between animals is not fully understood, although it is known that contact between infected and healthy animals can result in new infections. So far, there has been no evidence that CWD has crossed the species barrier in a similar manner to BSE; however, experimental transmission with CWD prions has successfully occurred in cattle(66). Preliminary serial passage experiments have demonstrated that CWD prions do not infect transgenic mice expressing the human prion protein, although this does not eliminate the possibility of infection after later passages, especially through an intermediate host such as cattle(67). With the known danger of human infection from BSE, and the potential for infection from the consumption of elk meat and venison, new, sensitive diagnostic tests for prion disease are necessary to ensure the safety of the food supply, as well as for the safety of the blood and organ transplant supplies.

There have been only three documented cases of BSE in the United States(68, 69). Although this number pales in comparison with the 186 000 confirmed cases in the UK, the US cases represent rare, and even novel, subtypes of BSE. The first case occurred in a 6.5 year old cow, imported from Canada, and was non-ambulatory when slaughtered in Washington state. Molecular and immunohistochemical analyses revealed similarities between this case, and preceding Canadian cases, as well as isolates from

European cases. Therefore, this case fits the profile of classical BSE, also called C-type, which accounts for the majority of BSE cases(69).

The second case of BSE diagnosed in the United States, affecting a 12 year old cow born and raised in Texas. This represents the first native case of BSE in the United States. This case differed from the typical histological and biochemical characteristics seen in C-type BSE. PrP^{Sc} was detected by immunohistochemistry, but the staining was much less intense than that in C-type BSE. The migration pattern determined by Western blot was different, as was the glycoform ratio. Significant sample enrichment was required in the Texas case to detect PrP^{Sc} in certain brain regions(69). Variability in the amount of brain tissue from the brainstem and cerebellum required for PrP^{Sc} detection indicates that PrP^{Sc} was not distributed uniformly in these regions. Unusual cases of BSE have been reported in several European countries, and those similar to the Texas cow have been designated "H-type", due to the unglycosylated band of PrP^{Sc} having a higher molecular mass than that of C-type BSE(69). The existence of rare forms of BSE suggest that there is more than one strain of BSE, with the unusual phenotypes perhaps representing a rare, sporadic form of the disease.

The third case of BSE identified in the United States affected a 10 year old beef cow in Alabama(68). Analysis of *PRNP* in this cow revealed a novel mutation, E211K, which had not previously been identified in cattle. This was of particular interest because the analogous mutation in humans, E200K, is the most common cause of fCJD. Therefore, the Alabama cow represents the first report of a potentially pathogenic mutation associated with a confirmed case of BSE(68). The phenotype was similar to that of the H-type BSE seen in the Texas cow described above; however, no mutation

was discovered in the Texas case. Epidemiological studies have determined that the prevalence of this mutation is very low, estimated to be less than 1 in 2000 cattle(70). However, it was found in the offspring of this cow, indicating that it is a heritable mutation that may persist in the cattle population. The discovery of atypical forms of BSE, that appear to be unrelated to the C-type epidemic that occurred in the UK, indicate that the need for BSE surveillance persists. It is of particular concern that many of the cattle diagnosed with H-type BSE in Europe showed no clinical signs of BSE at slaughter(69). With surveillance in the US only extended to "downer" cattle, the threat of BSE entering the human food chain still exists.

Although it is well documented that the spread of BSE is likely to have occurred due to the practice of feeding cattle meat and bone meal, the origin of the disease remains enigmatic. There are several theories, the first of which is that it originated with sheep scrapie contaminated cattle feed(71). However, the natural transmission of scrapie to cattle has not been demonstrated, and scrapie has been endemic in the UK for two centuries, with no prior evidence of transmission. A second theory is that BSE originated as a sporadic or genetic disease of cattle, which was then transmitted to other cattle with contaminated feed. Prior to the identification of the rare atypical forms of BSE, and the genetic mutation in the Alabama cow, there was no evidence that BSE naturally occurred in cattle(68). However, with the sheer volume of cases in the UK and Europe during the height of the epidemic, it is possible that any atypical case would have gone unrecognized. The final theory for the origin of BSE is that it originated from human prion diseases(71). Imports of fertilizer and animal feed from the Indian subcontinent have long been known to be contaminated with human remains, and if this was

incorporated into cattle feed in sufficient quantity, cattle may have become infected(71). Although the incidence of BSE has declined dramatically since the ban on the use of meat and bone meal in cattle feed, it has not been eliminated completely. There remains a possibility that horizontal transmission may occur, as is thought to be the case with CWD. Therefore, continued surveillance, and investigation into novel, more efficient diagnostic methods is of the utmost importance.

1.5 Current Prion Disease Diagnostics

At the present time, prion disease is definitively diagnosed through the detection of PrP^{Sc} in the brain or lymphoid tissue. Brain tissue is most often acquired at autopsy, although it is possible to perform a brain biopsy if CJD is suspected. The most convenient method currently available for the detection of PrP^{Sc} is the Western blot(72). This method requires the proteinase K (PK) digestion of brain homogenate. PK digestion takes advantage of the protease resistance of PrP^{Sc}, one of its biochemical hallmarks. Following the denaturation of the PK-resistant core of the prion protein, PrP(27-30) with an SDS buffer, a Western blot can be performed with one of many commercially available antibodies against the prion protein(72). For human samples, the monoclonal antibody 3F4, recognizing PrP residues 109-112, is currently the standard(72, 73). Western blotting is used because it shows a relatively high level of sensitivity, is easy to interpret, and does not require a large amount of tissue. Additionally, this method allows for observation of the migration differences between type 1 and type 2 CJD, and can provide extremely accurate diagnosis when used in combination with genetic studies to determine the codon 129 polymorphism and the presence of mutations

Western blotting is usually performed in combination with immunohistochemistry to provide the most reliable diagnosis(72). While Western blotting is adequate for detection of PrP^{Sc}, better anatomical resolution is provided by immunohistochemistry. This method provides information about the distribution of PrP^{Sc} among brain structures, and does not require fresh tissue. However, its sensitivity is not as consistent as Western blotting, and there can be problems with background reactivity from PrP^{C}. The latter issue can be overcome to a large extent with special processing steps, such as treatment of the tissue section with hydrolytic autoclaving(72).

1.5.1 Premortem Laboratory Diagnosis

In 1986, it was discovered that there were proteins present in the CSF of CJD patients, which were not present in healthy individuals, with sensitivity and specificity as high as 95%(74). These proteins were later identified as belonging to the 14-3-3 family, a group of highly conserved, multifunctional proteins highly expressed in neurons(75). They have been found to play an important role in cell signal transduction, proliferation, and differentiation. The finding of 14-3-3 in the CSF is indicative of acute neuronal damage. The Western blot is used for the detection of 14-3-3 proteins in the CSF, and the protein appears as a band of approximately 30 kDa. Although some studies found an overall sensitivity of 14-3-3 as a surrogate marker for CJD to be as low as 53%, such studies failed to account for differences in disease phenotype, nor was a consistent method of detection utilized(38). However, dividing cases of sCJD according to type 1 or 2 PrP^{Sc} and codon 129 zygosity found that the sensitivity varies among subtypes (Table 2).

Table 2. Six molecular subtypes of s CJD with corresponding sensitivity of the 14-3-3 test(38)

Sporadic CJD and subtypes	N	14-3-3 Test Sensitivity
sCJD (total)	90	87
MM1	48	94
MV1	3	100
VV1	3	100
MM2	10	70
MV2	7	57
VV2	19	84

The highest levels of sensitivity are found in certain subtypes of sCJD, especially in the most common molecular subtypes. MM1 and MV1 together account for approximately 70% of sCJD cases. 14-3-3 is also found in certain types of genetic CJD, most commonly in mutations E200K and V210I, but is not a reliable marker in fatal familial insomnia or GSS(76). It has a low sensitivity in iatrogenic CJD (60%) and in vCJD (48%)(77). Therefore, its utility as a surrogate marker is limited to the diagnosis of sCJD(76).

Elevated levels of 14-3-3 are not limited to CJD, as an indicator of acute neuronal damage, it is also found in other neurological conditions, such as stroke, hemorrhage, paraneoplastic disorders, cerebral neoplasias, and is found to be transiently elevated following an epileptic seizure(34). It is also found on occasion in other neurodegenerative diseases such as Alzheimer's disease, although this finding is relatively rare(78). Many of the conditions in which elevated levels of 14-3-3 are found differ from CJD in clinical presentation, or other neurological tests. However, because 14-3-3 is not definitively diagnostic of CJD, the results of this test must be interpreted carefully in the proper context.

The electroencephalogram (EEG) has been used since the 1950s in the diagnosis of CJD(76). In sCJD, an abnormal EEG pattern known as periodic sharp and slow wave complexes (PSWCs) is found, occasionally as early as 3 weeks after disease onset. PSWCs are seen within 12 weeks of disease onset in approximately 60%-70% of sCJD cases, as well as in genetic cases resulting from mutations in codon 200 and 210. The presence or absence of PSWCs in sCJD may be a result of codon 129 genotype, and whether type 1 or type 2 PrP^{Sc} is present(76). There is no diagnostic EEG pattern in any other type of prion diseases, including FFI, GSS, iatrogenic or variant CJD. Although there is no EEG pattern for vCJD, there are signal enhancements present on MRI, the most pronounced of these occurring in the posterior thalamus. This signal enhancement is called the pulvinar sign, and it is found in approximately 78% of vCJD cases(57, 76). As such, it has been included in the diagnostic criteria for the diagnosis of probable vCJD(34). Because the pulvinar sign is not present in sCJD, this represents an important distinction between sporadic and variant CJD. Similarly, the PSWCs are not found in vCJD, potentially providing a second method of distinction. Differentiation between these two types of human prion disease is essential from a public health perspective. However, because EEG and MRI signs, as well as elevated levels of 14-3-3 are not found in all cases of CJD, this distinction cannot be made conclusively prior to autopsy. Therefore, there is currently an urgent need for premortem test that can diagnose different types of human prion disease with high levels of sensitivity and specificity.

Variant CJD, as mentioned previously, is thought to arise from the consumption of beef contaminated by BSE(30, 39). As is observed in other instances of oral infection, prion replication and accumulation occurs in the spleen and lymphoreticular tissues prior

to invasion of the central nervous system. In animal models of scrapie, PrP^{Sc} may be detected in lymphoreticular tissues in approximately one third to half the usual incubation period(79, 80). Similarly, PrP^{Sc} was found in the tonsils in autopsy samples of individuals who had died of vCJD by both immunohistochemistry and Western blot(79). It is important to note that PrP^{Sc} is not found in the tonsil of patients with sporadic or inherited prion diseases, so the presence of PrP^{Sc} in the tonsil represents a way to differentiate vCJD from other types of human prion disease. The biopsy procedure itself is not without risk; however it is certainly much less risky than a biopsy of brain tissue. Living patients whose tonsils were biopsied made uneventful recoveries, and were able to resume normal diet without the need for pain medication by the first postoperative day(79). This recovery compares favorably with that from a full tonsillectomy procedure. The possible disadvantages of this method for widespread use as a screening tool are that not all individuals have sufficient amounts of tissue for an adequate biopsy. Tonsils are often removed surgically, and even when this is not the case, some individuals experience tonsillar atrophy(79).

Because PrP^{Sc} is found in the tonsils and lymphatic tissue of vCJD patients prior to nervous system invasion, it has been possible to detect PrP^{Sc} in tissues of individuals prior to the onset of clinical disease(58, 59, 80). It is through testing of lymphatic tissue at autopsy that an individual who had died of a ruptured aortic aneurysm was found to have PrP^{Sc} accumulation(61). This individual had not shown any symptoms of neurological disease prior to death. However, it was known that the patient had received a blood transfusion contaminated with vCJD five years prior to death. This finding was remarkable, because for the first time, evidence of vCJD infection was present in an

individual heterozygous at codon 129(61). While it is not known if this individual would have ever developed clinical disease, it raises concerns for iatrogenic transmission, especially as PrP^{Sc} in this case was not detected in the brain or spinal cord. While awareness exists of potential risks for prion contamination of neurosurgical instruments, the risk of transmission from surgical instruments used on the spleen or tonsils, both common surgical sites, may not be as obvious, especially in asymptomatic patients. A second possibility is that codon 129 heterozygous individuals are susceptible to vCJD, after a more extended incubation time than MM homozygotes. Both scenarios clearly illustrate the need for continued surveillance, and the development of sensitive diagnostic reagents.

The lack of a highly sensitive and specific diagnostic test, especially one that utilizes peripheral body fluids, represents the biggest limitation of current prion disease diagnostics. As mentioned previously, although there are some pre mortem tests which are highly suggestive of CJD, the disease can be present in the absence of these signs. Since the only reliable biomarker of prion disease is PrP^{Sc}, future efforts in the development of diagnostic tests must focus on novel ways to detect the protein in easily accessible body fluids pre mortem, or ideally, pre symptomatically. Attempts have been made to find PrP^{Sc} in the CSF, blood and urine. So far, there has been no conclusive evidence that PrP^{Sc} is present in any of these fluids, although PrP^{C} has been found in the blood and urine(81-84). If PrP^{C} is present under normal conditions, this suggests that PrP^{Sc} will be present during the disease. With the discovery of PK sensitive forms of PrP^{Sc} through such methods as the conformation-dependent immunoassay, it has been hypothesized that the PrP^{Sc} present in blood may be PK sensitive(24). Therefore, the

current methods for detecting PrP^{Sc}, reliant upon PK digestion for the elimination of PrP^{C}, would fail to detect PrP^{Sc} in this case. For this reason, reagents are needed that can detect PrP^{Sc} with a high level of sensitivity, in the absence of PK digestion.

Most commercially available antibodies against the prion protein recognize both PrP^{C} and PrP^{Sc}, because the primary sequence is identical. There have been several attempts to develop reagents specific for PrP^{Sc}, and many of these studies have had promising results. When developing PrP^{Sc} specific antibodies, most antibodies rely on the change in conformation, while others are against molecules other than PrP. Conformational antibodies are against epitopes that are thought to be buried in PrP^{C}, but are exposed upon the refolding of the protein to PrP^{Sc}. For example, the antibody 15B3 was found to have a conformational epitope, mapped to three polypeptide segments, the first two of which were found to be close together spatially in recombinant PrP, with the third located in another part of the molecule(85, 86). It was hypothesized that in the pathological conformation, all three segments are near neighbors, allowing binding of the antibody to the pathological conformation. The antibody against the YYR epitope, found to be specific for PrP^{Sc}, is thought to bind to tyrosine residues exposed on the surface of PrP^{Sc}, but are buried in PrP^{C}. The exposure of such hydrophobic residues on the surface is consistent with PrP^{Sc}'s insolubility and propensity for aggregation under physiological conditions(87). Finally, an antibody against the aggregated peptide PrP(106-126), called P1:1, was developed(88). This antibody bound to human prion protein from a variety of diseases, including vCJD. In immunopreciptation experiments, much like antibody 15B3, binding to intact PrP^{Sc} was more effective than binding to PK treated PrP^{Sc}. Again, this was thought to be as a result of a conformational epitope, and the lack of

binding to PK treated PrP^{Sc} was thought to be due to structural differences potentially masking the epitope in PK treated PrP^{Sc}. Such conformational epitopes cannot be confirmed, because the structure of PrP^{Sc} has not yet been solved. Therefore, it is not conclusively known which residues are exposed on the surface of the protein, how these differ from PrP^{C}, and in addition, whether there are any other molecules associated with PrP^{Sc}.

There has been a report of one antibody that is not against the prion protein, but is still able to reliably detect PrP^{Sc}, but not PrP^{C}, in samples of brain homogenate from a variety of prion disease, both human and animal(89). The antibody, called OCD4, was raised against DNA, and PrP^{Sc} binding to OCD4 is significantly diminished in the presence of DNA(89). It is unclear whether this binding is a result of DNA that is bound to PrP^{Sc} in vivo, DNA that becomes associated with PrP^{Sc} during the brain homogenization process, or a conformational similarity between PrP^{Sc} fibrils and the DNA epitope of OCD4. Treatment of PrP^{Sc} with DNase does not abrogate the binding of PrP^{Sc} to OCD4, suggesting that if DNA is bound to PrP^{Sc}, the DNA is likely inaccessible to the enzyme.

1.6 Limitations of Current Methods

The most significant limitation of the current diagnostic methods is that prion diseases cannot be conclusively diagnosed until autopsy. While there are many clinical signs, as well as the presence of surrogate markers such as the 14-3-3 protein, the disease can be present in the absence of many of these signs. In addition, the EEG and MRI signs can also be absent in the presence of histopathologically confirmed cases of

sCJD(90). Therefore, despite the progress in developing pre-mortem tests for the diagnosis of CJD, confirmation of the presence of PrP^{Sc} in brain tissue remains the only method by which the diagnosis can be confirmed. If the detection of PrP^{Sc} is the only way to definitively diagnose prion disease, then it is important that efforts in this area focus on novel ways to detect the protein pre-mortem, or ideally, pre-symptomatically. The least invasive way to accomplish this is through testing of peripheral fluids, such as blood, urine or CSF. PrP^{Sc} has not yet been detected in CSF(81). Although this may be a result of assay sensitivity rather than a true absence, it is currently not an appropriate fluid on which to base a diagnostic test. However, significant progress has been made in PrP^{Sc} detection experimentally in blood and urine. Despite this progress, no diagnostic test has been introduced that is based on either of these fluids.

1.6.1 PrP^{Sc} in blood

With the documentation of cases of vCJD resulting from blood transfusions, it is apparent that the blood contains infectivity(60). As mentioned previously, there is significant PrP^{Sc} accumulation in the lymphoreticular systems of vCJD patients, indicating a potentially important role of the lymphoreticular system in the pathogenesis of vCJD. Therefore, the lymphocyte fraction of the blood is thought to contain significant infectivity. Direct detection of PrP^{Sc} in the blood has been challenging, as it is not detectable by Western blot; however PrP^{C} has recently been found using a number of techniques including DELFIA and other highly sensitive ELISA-based assays(82, 83, 91). These assays suggest that blood borne PrP^{Sc} would be found primarily in the lymphocyte and platelet fractions of the blood, once the conformational change had taken place. In sheep naturally affected by scrapie, an increase of beta sheet content of the

membranous fraction was found using Raman spectroscopy(91). This is not a definitive finding of PrP^{Sc}, but it is suggestive of its presence. PrP^{Sc} has been detected in the blood of infected, but pre-symptomatic hamsters using the protein misfolding cyclic amplification technique, which will be discussed later(92). Again, however, this does not represent direct detection of PrP^{Sc}.

Although vCJD has been transmitted through blood transfusions, there have been no other cases of iatrogenic CJD that have arisen in this manner. The infectivity of the blood may be specific to vCJD due to differences in the pathogenesis of this disease compared to the other human prion diseases. The presence of infectivity in the blood of vCJD patients indicates that PrP^{Sc} should be detected given a sensitive enough assay. A blood test for vCJD would be minimally invasive, and have potential for use as a screening tool, which would be especially helpful in cases where exposure to PrP^{Sc} is known to have occurred. Based on the assumption that the majority of prion infectivity is contained in the leukocyte fraction, blood used for transfusion in the UK now undergoes leukocyte depletion. This is estimated to remove only 42% of the infectivity of the blood, indicating that it does exist in other fractions(93, 94). Although a large proportion of PrP^{C} was found in the platelet fraction, platelets are not found to be associated with infectivity in experimentally infected hamsters(95).

1.6.2 PrP^{Sc} in urine

There have been no reported incidences of CJD arising from contact with urine. However, as an easily accessible peripheral bodily fluid, the possibility exists that a

diagnostic test can be developed based on the discovery of PrP^{Sc} in urine. This has been a controversial idea, with no definitive findings of PK resistant PrP in the urine of infected animals. Infectious prions have been biochemically detected in the urine following the PMCA technique, similar to the experiments performed for PrP^{Sc} detection in blood(96, 97). Shaked et al (2001) initially reported finding protease resistant prion protein in the urine of scrapie infected hamsters, using the antibody 3F4(98). However, the PrP they discovered was not infectious upon intracerebral inoculation. Later studies demonstrated that urinary proteins are contaminated with bacterial membrane proteins, and the presence of immunoglobulins can be a source of confusion when using 3F4(99, 100). In either case, the proteins may be misidentified as PrP. Recent work has demonstrated that PrP^{C} is readily detectable in urine using antibodies against epitopes C-terminal to that of 3F4(84). This indicates that it may be possible to directly detect PrP^{Sc} under the right conditions, although this has yet to be confirmed. However, as a precaution, urine-derived gonadotropins are not recommended for use in reproductive procedures, such as in vitro fertilization(101). Like a blood test, a urine test for CJD would provide a minimally invasive method on which to base a diagnostic test.

1.7 Emerging Diagnostic Technologies and Detection Reagents

Highly sensitive studies of tissue distribution of PrP^{Sc} in cases of CJD revealed that the eye and optic nerve were PrP^{Sc}-positive in vCJD(102). This prompted Zanusso et al. to examine the olfactory sensory pathway for PrP^{Sc} in tissue derived from sCJD cases(103). Initially, the studies took place using autopsy samples from individuals with neuropathologically confirmed CJD. PrP^{Sc} was found with a Western blot in the olfactory epithelium of these individuals(103). The olfactory epithelium represents a

novel biopsy site for prion diseases, especially sCJD. This is significant, because the tonsillar biopsy described previously only detects PrP^{Sc} in cases of vCJD. A biopsy from the olfactory epithelium does not carry the risk of a brain biopsy, and the presence of PrP^{Sc}, as mentioned previously, would give a definitive diagnosis of the condition.

The nasal biopsy has also been performed on a patient presenting with suspected CJD, pre mortem, 45 days after the onset of symptoms(104). The procedure is performed under local anesthesia. PrP^{Sc} was detected in this case using immunohistochemistry, but not Western blotting. PrP^{C} is also present in the olfactory epithelium, and this is readily detectable in PK untreated samples(104). However, the amount of tissue required for PrP^{Sc} detection using a Western blot, especially pre mortem, is not practical as a biopsy sample(104). Because the Western blot is more informative for PrP^{Sc} typing, this is a significant disadvantage. Nevertheless, the ability to detect PrP^{Sc} with immunohistochemistry suggests that the olfactory biopsy is a promising candidate for confirmation of a CJD diagnosis, even pre-mortem(104).

A second method by which PrP^{Sc} may be differentiated from PrP^{C} is the conformation-dependent immunoassay, first developed for the examination of prion strain differences(24). This assay is highly sensitive with the recent adaptation of DELFIA techniques. This method is similar in style to a conventional ELISA, so it is amenable to high throughput screening, greatly facilitating the testing of a large population of individuals. One method of performing a conformation-dependent immunoassay involves denaturing PrP in increasing concentrations of guanidine HCl. PrP^{Sc}, because of its conformation, is resistant to denaturation especially at lower concentrations of guanidine(24).

Protein misfolding cyclic amplification (PMCA) was first described by Soto and colleagues in 2001(105). Conceptually similar to PCR, PMCA uses minute quantities of PrP^{Sc} as a seed, and incubates it with large quantities of PrP^{C}. The PrP^{C} serves as the source for conversion into PrP^{Sc} and the formation of PrP^{Sc} aggregates, which are then sonicated to break them up into smaller seeds, for the continued formation of more PrP^{Sc}(105). The sonication step is not essential, but the efficiency is much improved when it is included. The extent of conversion depends on the number of PMCA cycles, and conversion is enhanced with the addition of small fragments of RNA(106). PMCA has tremendous diagnostic potential, due to its ability to amplify minute quantities of PrP^{Sc}. It has been successfully used to detect PrP^{Sc} in the blood of experimentally infected hamsters, with 89% sensitivity and 100% specificity(107). In addition, PMCA has been used to detect PrP^{Sc} in the brain of pre-symptomatic animals, which are negative by Western blot and immunohistochemistry(92). Finally, PMCA has also been used for the detection of PrP^{Sc} in the urine of infected animals, estimated to be present at concentrations 10-fold lower than blood(96). Due to the sensitivity of this technique, PMCA appears to be an excellent candidate for the development of diagnostic tests for the pre-symptomatic period, or that utilize peripheral fluids.

Recent work from Deleault and colleagues may compromise the diagnostic utility of PMCA(108). Using a minimal set of components, including native PrP^{C}, copurified lipid molecules and a synthetic polyanion, infectious PrP^{Sc} was generated with no pre-existing seed. These spontaneously generated prions were inoculated into hamsters, resulting in disease which was transmissible on second passage(108). While spontaneously generated prions, and the components necessary, have implications for

prion biology in general, it also calls into question the use of PMCA in diagnostic situations in which very small amounts of PrP^{Sc} is present(108). Lipids, PrP^{C} and polyanions are present in most body fluids, and it is vital that prions are not formed *de novo* from these components in the course of a diagnostic test. It has been suggested that PMCA is only capable of spontaneously generating PrP^{Sc} under certain experimental conditions, and that adjusting the parameters of PMCA can ensure that this does not occur(29). However, until this can be conclusively settled, investigations into alternate diagnostic technologies must continue.

CHAPTER 2

CRYPTIC PEPTIDES OF THE KRINGLE DOMAINS PREFERENTIALLY BIND TO DISEASE-ASSOCIATED PRION PROTEIN

SUMMARY

Prion disease is a group of fatal neurodegenerative diseases that are characterized by the accumulation of misfolded scrapie form (PrP^{Sc}) of the normal cellular prion protein (PrP^{C}) in the brains of affected individuals. The conversion of PrP^{C} to PrP^{Sc} is thought to involve a change in protein conformation from its normal, primarily α-helical structure into a β-sheet conformer. Few proteins have been identified so far that differentially interact with the two forms of PrP. Previously, it has been reported that the full-length plasminogen protein, binds to PrP^{Sc} from a wide variety of prion phenotypes, including Creutzfeldt-Jakob disease and bovine spongiform encephalopathy. A recent study has shown that recombinant mouse PrP, both in α-helical and β-pleated conformations, interact with the kringle domains of plasminogen *in vitro*. In the present work, we have examined potential motifs within the kringle region that may be responsible for binding to PrP. We synthesized 12-15-mer peptides that contain small stretches of amino acid residues found within the kringle domains of plasminogen. We show that these small peptides have high affinity for binding to PrP^{Sc}, but not PrP^{C}. These synthetic peptides were found to capture PrP^{Sc} from the brain homogenates of BSE affected cattle, CWD affected elk, hamsters experimentally infected with the 263K strain of scrapie as well as from that of subjects affected by Creutzfeldt-Jakob disease, without

binding to PrP^{C} in unaffected controls. Therefore, we have identified critical peptide motifs that may be important for protein-protein interactions in prion disease pathogenesis. Furthermore, the ability of these peptides to bind preferentially to PrP^{Sc} suggests a potential application in the diagnosis of prion disease.

INTRODUCTION

Plasminogen is a ubiquitous pro-protease which may be cleaved to form the serine protease plasmin, an important component of the fibrinolytic system(109). The fibrinolytic pathway has been implicated in biological processes as diverse as cell migration(110), inflammation(111), neuronal plasticity(112) and embryonic implantation(113), and also excitotoxin-mediated neurotoxicity(114) or Alzheimer's disease(115). Plasminogen may be activated by one of two mechanisms, the urokinase plasminogen activator (uPA) and the tissue type plasminogen activator (tPA)(116). The former is normally associated with pericellular proteolytic activity, while the latter is associated with the dissolution of intravascular clots(109). It has previously been demonstrated that plasminogen forms a complex with PrP^{Sc} from mice, and a variety of other species, with no binding observed to PrP^{C}(117, 118). This interaction was thought to be mediated through the first three Kringle domains of plasminogen(117). Each Kringle domain is composed of approximately 80 amino acids, and contains 3 disulfide bonds(119). They are joined to other Kringle domains by varying lengths of linker amino acids. Kringle domains are not unique to plasminogen; they are also found in a variety of proteins associated with fibrinolysis, coagulation and angiogenesis(119). The first three Kringle domains in plasminogen contain a lysine binding domain, which may be responsible for the majority of its interactions with other proteins(120). Structurally, plasminogen contains five Kringle domains in total, as well as a C-terminal proteolytic domain. Although no interaction between plasminogen and PrP^{C} was found using

plasminogen to capture PrP^C from brain homogenate(117, 118), later work indicated the possibility of an interaction with recombinant human PrP^C(121-123). While there has been no evidence of an interaction between plasminogen and PrP^{Sc} or PrP^C *in vivo*, *in vitro* there have been several recent studies that indirectly suggest an interaction using both recombinant human PrP^C, as well as investigations into the role of plasminogen in CJD(124).

Binding of recombinant human PrP^C has been observed to both plasminogen and tPA using surface plasmon resonance(121). Direct binding of full length recombinant PrP to the Kringle domains of plasminogen was observed by ELISA, but was significantly reduced using the recombinant fragment PrP(89-231) (122). Recombinant prion protein, bound to copper, was found to increase the rate of activation of plasminogen with tPA, but no effect was found in the absence of copper, or with the uPA activation system(123). This activation of plasminogen using PrP was found to be conserved in the N-terminal region of the protein, which is a relatively unstructured region to which copper was known to bind(121, 125). Further, plasmin was found to cleave PrP^C, with the cleavage site located at lysine 110, and that the activation was further augmented in the presence of low molecular weight heparin(126). Mutants of PrP were made in later work by the same group, and it was then discovered that both lysine clusters in the N terminal region of the prion protein are required for activation(127). Again, the activation of the plasminogen was only found to be enhanced in the presence of PrP^C in the tPA system.

The cortical neurons of individuals with CJD have been found to express the uPA receptor(124). The expression of this receptor is thought to be associated with signaling

cascades that lead to eventual neurodegeneration. Although neurons associated with the most uPA receptor had the most degenerative hallmarks, no interaction was demonstrated between the plasminogen itself and either form of the prion protein. When plasminogen concentrations and activities were examined in brains of patients with CJD, compared to those with other forms of dementia, it was found that the plasminogen concentrations were higher, but the activity was lower in CJD(128). This suggests a relationship between plasminogen and the disease process of CJD, but it is not conclusive.

An initial report suggested that tPA activity and gene expression were elevated in the brains of scrapie-infected mice, and that mice deficient in plasminogen, as well as those deficient in tPA experienced shorter incubation periods and a more rapid disease course than scrapie-infected wild type mice(129). Further, plasmin was found to degrade PrP^{C}, in agreement with previous studies, but did not degrade PrP^{Sc}. This led the authors to conclude that plasminogen may have a neuroprotective role in prion disease(129). However, at variance with those authors, a second study concluded that plasminogen plays no role in scrapie(130). While PrP^{Sc} accumulation in the brains and spleens of scrapie-infected mice, as well as more severe neuropathological changes, were found in the early stage of disease in the wild type mice compared to plasminogen knock out mice, these differences were no longer present by the terminal stage of the disease(130). In the absence of scrapie infection, plasminogen knock out mice were investigated for differences in the cellular processing of PrP^{C}, based on its degradation by plasmin in vitro studies. Plasminogen deficient mice did not demonstrate altered cleavage of PrP^{C}, suggesting that plasmin plays no significant role in PrP^{C} processing in vivo(131).

With the discovery of the possibility of a causal relationship between the consumption of beef contaminated with BSE and the development of vCJD, prion diseases for the first time presented a challenge for public health (7, 30). At the present time, diagnosis of the disease is limited to the detection of proteinase K (PK) resistant PrP^{Sc} in a Western blot, or by immunohistochemistry, both of which are usually performed on brain tissue at autopsy(72). We aimed to exploit the PrP^{Sc} binding properties of the plasminogen Kringle domains through the development of short peptides, based on sequences that appear frequently within the plasminogen Kringle domains. These peptides bind to PrP^{Sc}, but not PrP^{C}, as detected by Western blot. Of several peptides that were found to bind PrP^{Sc} in preliminary analysis, we selected two for further study, one 12 residues in length, designated peptide 1 (P1), (sequence: YRGYRGYRGYRG), and the second, 15 residues in length, designated peptide 2 (P2), (sequence: YRGRYGYKGKYGYRG).

METHODS

Reagents and Peptides—Magnetic beads (M-280 streptavidin) were from Dynal (Oslo, Norway). Proteinase K (PK) was obtained from Sigma, and Pefabloc SC (4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride) protease inhibitor was obtained from Roche Applied Science (Indianapolis, IN). Antibodies used in this study were 3F4, recognizing an epitope of human PrP at residues 109-112 (73) and animal prion protein was detected using the monoclonal antibody 8H4, which recognizes human PrP at residues 145-180(132). The horseradish peroxidase secondary antibody, and the chemiluminescence reagent were both purchased from Amersham Biosceinces (Piscataway, NJ). All other chemicals and reagents were purchased from Sigma, unless otherwise indicated. Peptides were synthesized using standard methods, obtained from Invitrogen (Carlsbad, CA). The sequence of P1 is NH2-YRGYRGYRGYRG[K-IcBiotin][CONH2], P2 is NH2-YRGRYGYKGKYGYRG[K-IcBiotin][CONH2], and the unrelated control peptide used was NH2-[biotin][AMCAP]-SEIKLLIS[CONH2].

Brain Tissues—Brain tissue was acquired at autopsy from individuals with and without prion diseases, and archived frozen at -80 ºC at the National Prion Disease Pathology Surveillance Center. The tissue was then homogenized in lysis buffer (100 mM NaCl, 10 mM EDTA, 0.5 % Nonidet P-40, 0.5 % sodium deoxycholate, 10 mM Tris HCl, pH 7.5) containing a mixture of protease inhibitors (Roche Applied Science) to a concentration of 10 % (w/v). The brain homogenate was stored at -80 ºC. The diagnosis of the various phenotypes of prion diseases was confirmed using standard methods, including immunohistochemistry, immunoblotting, and DNA typing. Animal brain tissues were

homogenized and stored in the same manner, with the diagnosis confirmed by immunoblotting and immunohistochemistry.

Peptide Capture Assay—The biotinylated P1 or P2 (70 µl of a 1 mg ml^{-1} preparation) was conjugated to $2.3x10^8$ streptavidin superparamagnetic beads in 1 ml of phosphate-buffered saline, pH 7.5 (PBS) at 37 ºC for 20 h. The conjugated beads were then incubated in 0.1 % BSA in PBS, at 37 ºC for 4 h, to block nonspecific binding. Once sodium azide is added (0.1 % final concentration), the conjugated beads are stable at 4 ºC for at least 8 weeks. The angiostatin (K(1+2+3)) were obtained from Sigma, and conjugated to $2.3x10^8$ tosyl-activated paramagnetic beads in 1 ml of PBS for 20 h at 37ºC. The conjugated beads were incubated in 0.1 % BSA 0.2 M Tris-HCl, pH 8.6, for 4 h at 37 ºC, to deactivate any unbound tosyl groups, and to block non-specific binding. The beads are stored at 4 ºC, in 0.1 % BSA in PBS. The peptide capture assay was performed using 100 µl of the conjugated beads in 900 µl of capture buffer (PBS, 3 % Tween 20, 3 % Nonidet P-40), to which the 10 % brain homogenate is added (6 µl of human brain homogenate, or 3 µl of animal brain homogenate). The mixture was incubated with constant rotation at room temperature for 3 h. Following the incubation, the beads were washed 3 times with wash buffer (PBS, 2 % Tween-20, 2 % Nonidet P-40), to remove unbound materials. The beads were then resuspended in SDS Sample buffer (3 % SDS, 2 mM EDTA, 10 % glycerol, 2.5 % β mercaptoethanol and 50 mM Tris HCl, pH 6.8), and heated to 100 ºC for 10 min.

Immunoblotting—The samples were loaded on to an SDS-PAGE gel (15 % Tris-glycine pre-cast gel, BioRad), separated, and electrotransferred onto a polyvinylidene difluoride membrane (Millipore). PrP was detected using the anti-PrP monoclonal antibody 3F4

(1:50,000) for hamster and human forms of the protein, and monoclonal antibody 8H4 (1:3,000) for other animal forms. Following the addition of a horseradish peroxidase conjugated secondary antibody, the ECL Plus kit (Amersham Biosciences) was used to visualize immunoreactivity.

PK Digestion—PK was added to samples of total brain homogenate, or conjugated beads resuspended in 20 µl of lysis buffer, at 50 µg ml^{-1}. Incubation, with shaking, was at 37ºC for 1 h. Pefabloc SC (Roche Applied Science, Indianapolis, IN) was added, at a final concentration of 5 mM, to stop the reaction. 2X SDS sample buffer was then added, before immunoblotting.

Conformational Stability Immunoassay— Increasing concentrations of guanidine HCl (final concentration 0-3M) was added to 10% total brain homogenate of scrapie infected hamsters or from CJD. The samples were incubated for 1 hour at room temperature. A five fold volume of pre-chilled methanol was then added, followed by centrifugation for 30 minutes at 4°C. The supernatant was discarded, and the pellet resuspended in lysis buffer, with the aid of sonication. PK digestion, or a peptide capture assay was then performed as described above.

Competition Assay. The competition assay was performed in a similar manner to the peptide capture assay described above. Increasing amounts of free peptide (P1, 0-100 µg) were added to each peptide capture reaction, and incubated at room temperature for 3 h, before immunoblotting as described above.

Plasma spiking assay. Plasma samples containing 80 mM EDTA from individuals unaffected by CJD were obtained from the National Prion Disease Pathology Surveillance Center. The peptide capture assay was performed as described previously,

with the capture buffer replaced with a solution of 900 µl plasma and 100µl capture buffer, followed by immunoblotting as described above.

RESULTS

The mini-Kringle peptides 1 and 2 specifically capture PrP^{Sc}, but not PrP^{C}. The mini-Kringle peptides were derived from the YRG and YRK sequences of peptides that appear frequently through out the sequence of the five Kringle domains found in plasminogen (Figure 2.1). Biotinylated peptides were conjugated to streptavidin magnetic beads, and incubated with 10% brain homogenate for 3 hours at room temperature. PrP was successfully captured in large quantities from brains of humans affected by prion disease. No PrP was captured from human brains unaffected by prion disease (Figure 2.2). To ensure that the binding of PrP to the peptides was specific, streptavidin beads without conjugated peptide were used, blocked with 0.1% BSA as in the normal procedure, and beads conjugated with an unrelated peptide were also tested. No PrP was captured with the unrelated peptide. A small trace amount of PrP was seen following prolonged exposure of the Western blot with the unconjugated beads, from the brains of individuals with CJD, but not from unaffected control brains (Figure 2.2). This effect may be due to the highly aggregated nature of PrP in the disease state.

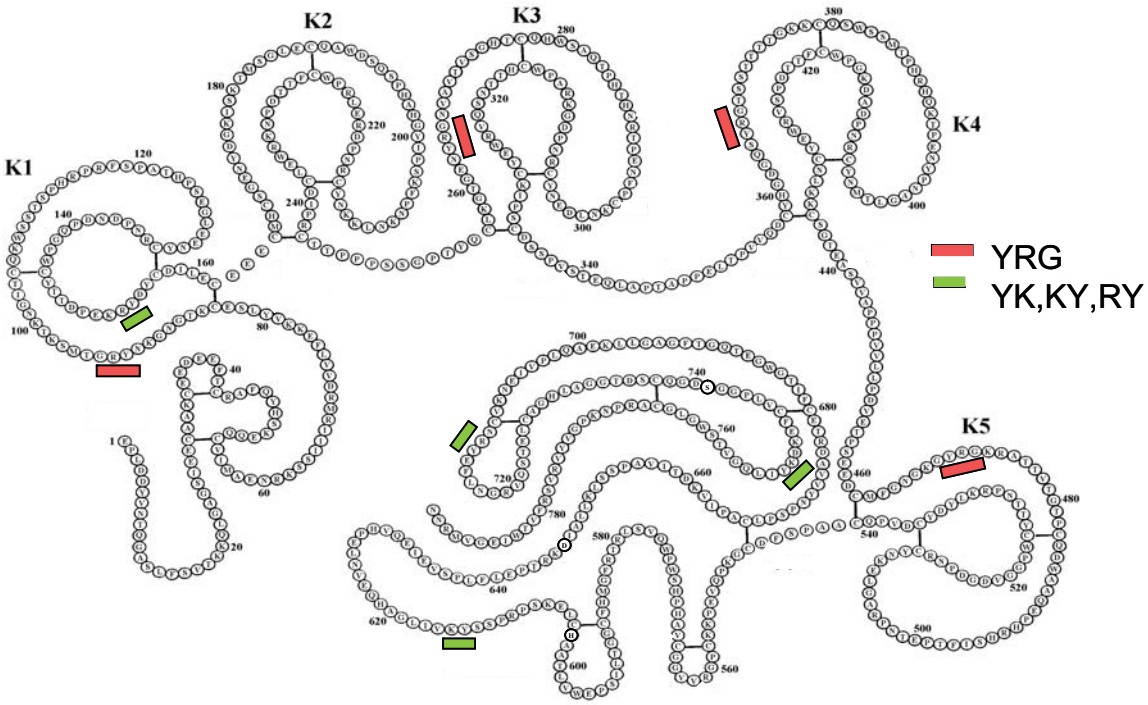


Figure 2.1. The presence of the YRG motif in the kringle domains of plasminogen. Plasminogen molecule consists of a total of five kringle domains labeled as K1-K5, and a C-terminal region. The YRG sequence appears in four out of the five kringle domains. The shorter YK, KY and RY sequences appear also in different regions of the plasminogen molecule.

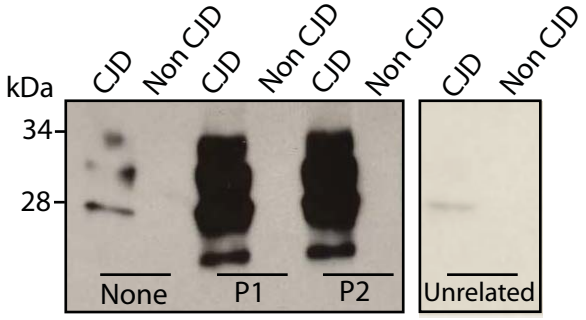


Figure 2.2 Capture of disease-associated PrP by P1 and P2. A. P1 and P2, biotinylated and coupled to streptavidin magnetic beads, were used to capture PrP from the brain homogenates of CJD patients, as well as unaffected controls (Non-CJD). Beads with no conjugated peptide, but blocked with 0.1 % BSA, were used as a negative control (None). An unrelated peptide was biotinylated and conjugated to streptavidin magnetic beads, as a second negative control (Unrelated).

The mini-Kringle peptides capture PrP from a variety of human and animal prion diseases. The peptide capture assay using peptides 1 and 2 was used to capture PrP from a wide variety of human and animal prion diseases, including the animal prion diseases with the greatest significance for human health, chronic wasting disease and bovine spongiform encephalopathy (Figure 2.3). The peptides were successfully captured PrP from these diseases, as well as from Syrian hamsters affected with the experimental scrapie strain 263K, but not from the brain of any animal unaffected by prion disease (Figure 2.3). In addition, the peptides successfully captured PrP from brain homogenate of patients affected by type 1 and type 2, as well as a case of mixed type 1/2 of sporadic CJD(4) (Figure 2.4A). Also tested were a genetic CJD case (E200K mutation), a case of GSS (P102L mutation), and CJD cases with an infectious etiology, iatrogenic and variant CJD (Figure 2.4). In all cases, brains from individuals who were unaffected by CJD were included. PrP was never captured from these brains, although it is abundantly present prior to capture by peptides 1 and 2 (Figure 2.4).

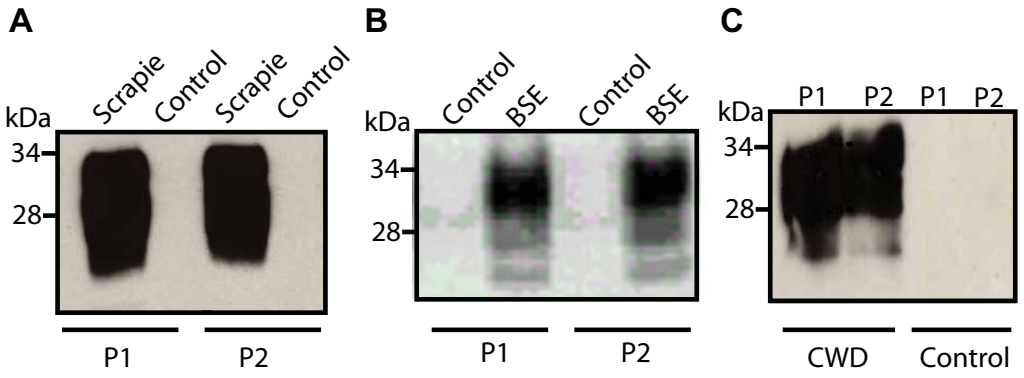


Figure 2.3. Capture of PrP^{Sc} from animal forms of prion disease by Peptides 1 and 2. P1 and P2, coupled to the magnetic beads, were used to pull down PrP^{Sc} in brain homogenates from Syrian hamsters infected with scrapie strain 263K (A.), cattle affected by BSE (B.) and elk affected by chronic wasting disease (C.). For the capture assay, peptide conjugated beads were incubated with 6 µl of 10 % brain homogenate in 1 ml PBS containing 3 % Tween-20 and 3 % NP-40, for three h at room temperature. Beads were recovered by applying magnetic field and proteins bound to beads were subjected to immunoblotting using either 3F4 (in A) or 8H4 (in B).

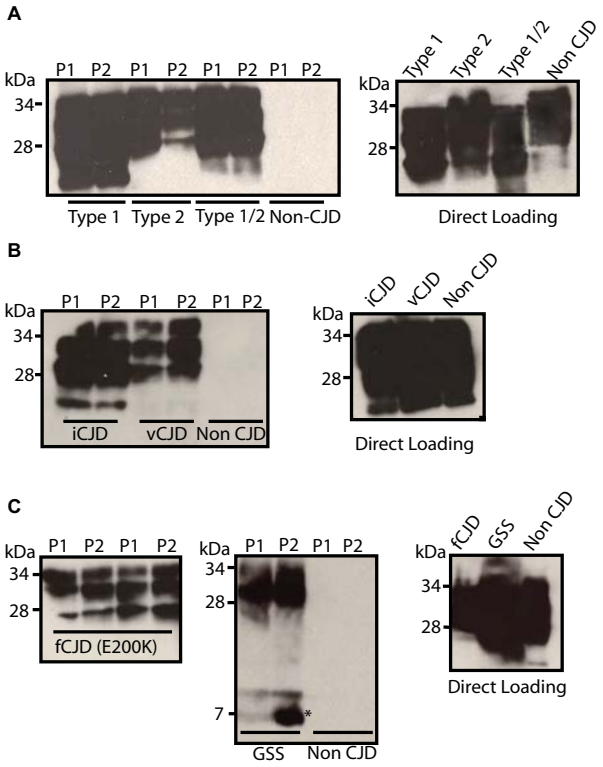


Figure 2.4. P1 and P2 capture PrP^{Sc} from different subtypes of human prion diseases. A. P1 and P2, coupled to streptavidin magnetic beads, were used to pull down PrP^{Sc} from 10% brain homogenate of individuals affected by Type 1 sCJD (Lanes 1 and 2), Type 2 sCJD (lanes 3 and 4), Type 1/2 sCJD (Lanes 5 and 6), as well as a CJD unaffected control (lanes 7 and 8), accompanied by a direct loading control of the same cases (Direct Loading). B. P1 and P2 were used to capture PrP^{Sc} from 10% brain homogenates of individuals affected by iatrogenic (lanes 1 and 2) and variant (lanes 3 and 4) CJD, as well as a CJD unaffected control (lanes 5 and 6), accompanied by a direct loading control of the same cases (Direct Loading). C. P1 and P2 were used to capture PrP^{Sc} from 10% brain homogenate from cases of familial CJD (E200K) and GSS. The 7kDa internal fragment of GSS (in C) is indicated by an asterisk. Immunoblotting was performed using 3F4.

PrP^{Sc} from humans and animals captured by the peptides is PK resistant. One of the most important biochemical characteristics of PrP^{Sc} is its resistance to PK digestion, and this feature is the basis for distinguishing PrP^{Sc} from PrP^{C} in current diagnostic tests. In order to determine whether the PrP captured by the beads was PK resistant, it was incubated in the presence of PK (50μg/mL). Samples used included beads incubated with homogenate from a Syrian hamster infected with experimental scrapie strain 263K, as well as homogenate from human cases of both type 1 and type 2 sporadic CJD. Previous work has shown that on an SDS-PAGE gel, the migration of the core PK resistant fragment of PrP^{Sc} differs between type 1 and type 2 cases of sporadic CJD(4). The same difference in migration is seen in the two cases of sporadic CJD following capture by Peptides 1 and 2 followed by PK digestion (Figure 2.5A). This indicates that the PrP binding to the peptides is PK resistant, with similar characteristics to those expected of different types of prion disease.

The peptides bind to the PK-resistant core C-terminal fragment of PrP^{Sc}. The peptides were also used to capture PrP that had previously been digested with PK. The fragment of PrP^{Sc} produced by PK digestion corresponds to a C terminal fragment of the protein, commonly referred to as PrP(27-30)(1). After incubation with peptides 1 and 2, the C-terminal fragment of PrP^{Sc}, PrP(27-30), was captured (Figure 2.5B). This suggests that the peptides interact with PrP^{Sc} in the core C-terminal PK resistant fragment, PrP(27-30). This fragment in PrP^{Sc} type 1 spans residues 82-231, and in type 2, 97-231(37, 72). Again, when cases of type 1 and type 2 sporadic CJD were used, the expected difference in migration was observed.

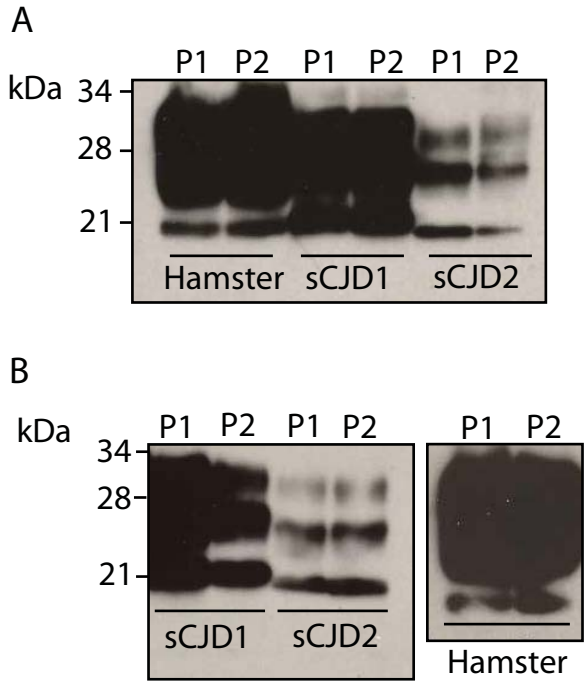


Figure 2.5. Binding of P1 and P2 to the PK-resistant core fragment of PrP^{Sc}. A. Total brain homogenate from a scrapie-infected hamster (Hamster) or sporadic CJD type MM1 (sCJD1) and VV2 (sCJD2), was captured by P1 and P2 coupled to streptavidin magnetic beads, followed by digestion with 50 μg/ml PK for 1 h at 37°C and immunoblotting with 3F4. B. Total brain homogenate of sporadic CJD type MM1 (sCJD1) and VV2 (sCJD2), as well as scrapie-adapted hamster (Hamster) was digested with 50 μg/ml PK for 1 h at 37 °C, prior to capture by P1 and P2. Immunoblotting was performed using 3F4.

Peptide binding to PrP^{Sc} is conformation dependent. When PrP^{Sc} is denatured using guanidine hydrochloride, as the concentration of the denaturant increases, PrP^{Sc} undergoes a conformational change. This property can be exploited to determine the conformational dependence of the interaction between PrP^{Sc} and another molecule. The amount of PrP captured with increasing concentrations of guanidine hydrochloride decreased indicates that the binding of PrP^{Sc} to the peptides is dependent on the conformation of the PrP. As a result, PrP^{Sc} derived from hamster scrapie (263K) loses its PK resistance at a concentration of 2.7M guanidine HCl (Figure 2.6A). Furthermore, the binding affinity between PrP and peptide 1 is decreased as the native conformation of PrP^{Sc} is lost (Figure 2.6B). However, approximately 20% of the original amount PrP^{Sc} is bound to peptide 2 at a guanidine concentration of 2.7M. This indicates that peptide 1 binds a small amount of PK sensitive PrP^{Sc}. This finding is consistent with other work, including conformation dependent and other analyses, that have previously indicated that PrP^{Sc} does contain PK-resistant and PK-sensitive species(33).

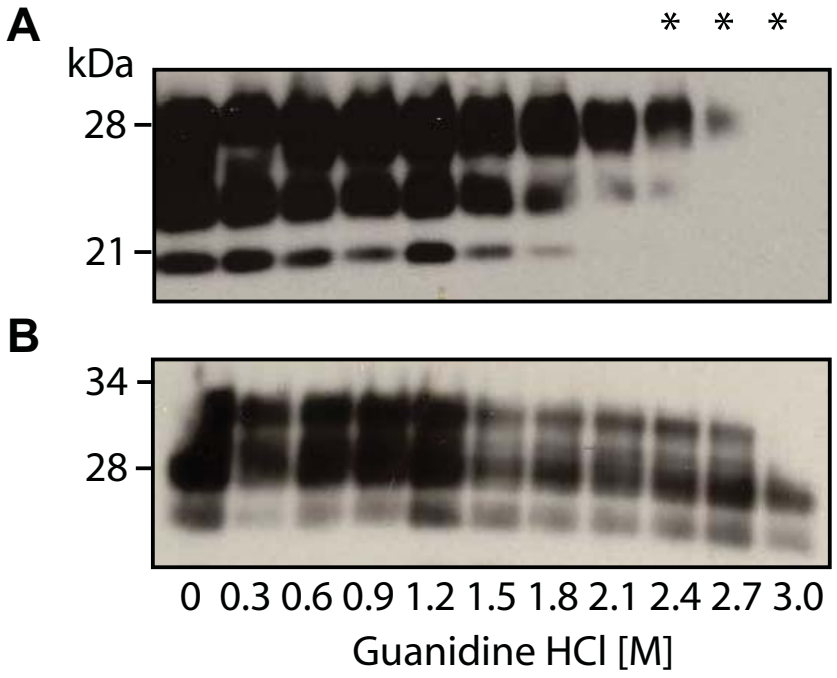


Figure 2.6. The binding of PrP^{Sc} to P1 is conformation dependent. A. Brain homogenate from scrapie-adapted hamsters was incubated for 1 h with guanidine HCl (0-3 M), followed by digestion with 50 μg ml^{-1} PK for 1 h at 37 °C and immunoblotting with 3F4. B. Brain homogenate from scrapie-adapted hamsters was incubated for 1 h with guanidine HCl (0-3 M), followed by incubation with Peptide 1 conjugated beads and immunoblotting with 3F4. The asterisks indicate where there is a difference between PK-resistant PrP and the PrP captured directly by P1.

The peptides capture PrP^{Sc} with similar affinity to the full length Kringle domains. We chose to compare the binding of PrP to the mini-Kringle peptides to the full length K(1+2+3) previously documented to bind to PrP^{Sc}. Approximately $2.3x10^7$ beads were used for each individual reaction, from the stock solutions of $2.3x10^8$ beads. Each individual reaction contained approximately 7 μg of peptide 1, and 50 μg of K(1+2+3). The peptide capture assay was performed with decreasing amounts of brain homogenate (3-0.5μL) from an experimentally infected Syrian hamster. An equimolar comparison was not made, due to the difference in molecular weight between K(1+2+3) and peptide 1, and because any differences between the stoichiometry of PrP^{Sc} binding to either K(1+2+3) or the peptides are not known. Nevertheless, there was negligible difference in the amount of PrP^{Sc} captured by K(1+2+3) and peptide 1, even using the smallest amount of brain homogenate (Figure 2.7). Therefore, the affinity of PrP^{Sc} for peptide 1 and K(1+2+3) appears to be comparable.

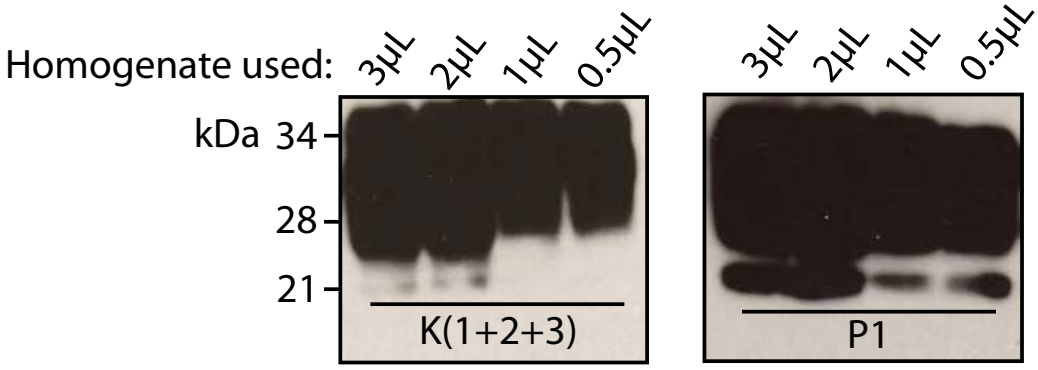


Figure 2.7. Binding of P1 to PrP^{Sc} compared to binding of angiostatin (K(1+2+3)).

A. Decreasing quantities of 10 % brain homogenate from scrapie-adapted hamsters (3 µl to 0.5 µl) were incubated with 50 µg of K(1+2+3) conjugated to $2.3X10^7$ tosyl-activated beads or 7 µg of P1 conjugated $2.3X10^7$ streptavidin beads. The mixtures were incubated for 3 hours at room temperature, followed by immunoblotting with 3F4.

Peptide 1 successfully competes with K(1+2+3) for PrP^{Sc} binding. Because the sequence of peptide 1 is derived from recurring residues in the Kringle domains, a competition assay was used to assess whether the binding site was shared. In a peptide capture assay, in which increasing amounts of free peptide 1 was added to the reaction, the amount of PrP^{Sc} bound to the K(1+2+3) conjugated beads decreased as increasing amounts of free peptide 1 were added (Figure 2.8). This indicates that free peptide 1 is able to bind to PrP^{Sc}, and that such binding is sufficient to inhibit the association between K(1+2+3) and PrP^{Sc}. While the binding site for this interaction has not been determined, the ability of peptide 1 to successfully compete with K(1+2+3) for PrP^{Sc} binding suggests that the binding properties of these two molecules may be similar.

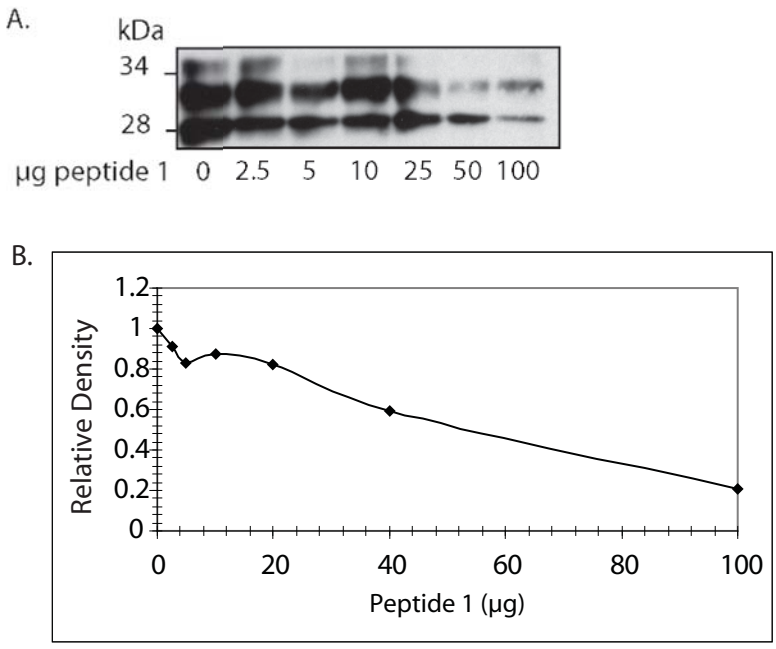


Figure 2.8 Peptide 1 successfully competes with K(1+2+3) for prion binding. A. Angiostatin (K(1+2+3), 1.7 mM) was conjugated to tosyl-activated magnetic beads and incubated with 4 µl of 10 % brain homogenate from a case of sporadic CJD. The binding of (K(1+2+3) to PrP was competed with increasing amounts (2-100 µg) of P1, followed by immunoblotting with 3F4. B. Computer aided densiometric analysis of A.

Substitution of the arginine residue of peptide 1 eliminates PrP binding. In order to determine which residues of peptide 1 were important for its interaction with PrP^{Sc}, several new peptides were synthesized, with substitutions made for the tyrosine or arginine residues (Figure 2.9A). When the peptide capture assay was performed using the substituted peptides, we found that peptides in which the arginine residue had been eliminated no longer bound PrP. This was true in both a peptide in which the arginine had been substituted for a negatively charged residue (aspartic acid) and a peptide in which it had been substituted for the neutral residue alanine (Figure 2.9B). However, when the arginine was retained, and the tyrosine was substituted, the peptide maintained its ability to capture PrP. This was true regardless of whether tyrosine was substituted for another aromatic residue, or whether the aromatic character was eliminated (Figure 2.9B). This indicates that the most important residues in the interaction with PrP are the positively charged residues such as arginine and lysine.

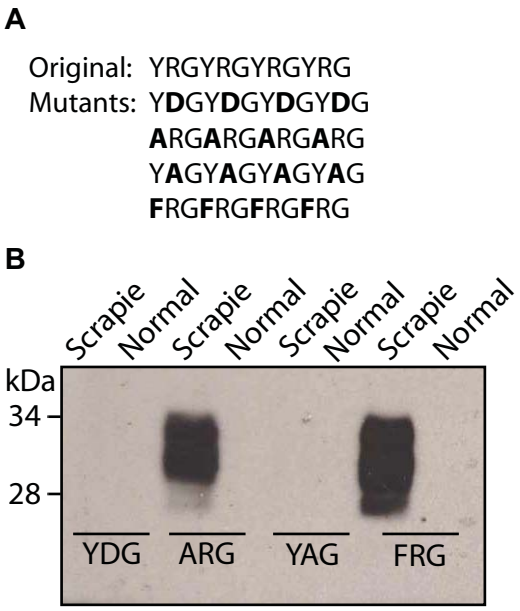


Figure 2.9. Effect of substitution of arginine and tyrosine residues on the binding of P1 to PrP^{Sc}. A. The sequences of P1 and the four substituted peptides. B. The substituted peptides were conjugated to streptavidin magnetic beads and were incubated at room temperature for 3 h with 3 µl of brain homogenate from scrapie-adapted hamster, or 6 µl of brain homogenate from a normal hamster. Immunoblotting was performed using 3F4.

P1 captures spiked PrP^{Sc} from human plasma—Because all of our experiments described above are based on capture of PrP^{Sc} from brain homogenates, it is not yet known whether such an assay will work for PrP^{Sc} in blood, in which kringle domain containing proteins such as plasminogen are present in high concentrations. This issue will likely affect the potential for a blood-based assay for PrP^{Sc} using kringle based peptides. As a first step, we tested the ability of P1 to capture spiked PrP^{Sc} from human plasma. Plasma was obtained from six different individuals unaffected by prion diseases, and was spiked with either 3 µl of 10% brain homogenate from hamsters infected with the 263K strain of scrapie, or from wild type hamsters. Following addition of P1 conjugated beads, capture reaction was carried out in either 1 ml of capture buffer, or in 1 ml of 90% of human plasma (900 µl of plasma and 100 µl capture buffer). Results from experiments in two different plasma samples showed that P1 successfully captured spiked PrP^{Sc} from the plasma (Figure 2.10A). This indicates that P1 readily captures PrP^{Sc} and other proteins present in plasma do not appear to significantly inhibit the P1 interaction with PrP^{Sc}, at least *in vitro*. No prion protein was captured from the plasma spiked with wild type brain homogenate (Figure 2.10A). The presence of input PrP^{Sc} and PrP^{C} in spiking sources of scrapie-infected and normal animals, respectively, was demonstrated by immunoblotting and PK digestion (Figure 2.9B).

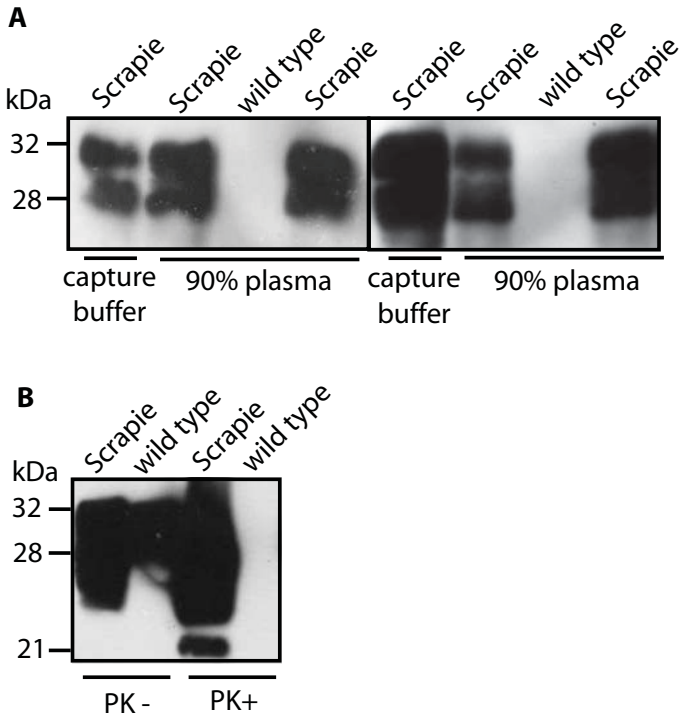


Figure 2.10. Capture of spiked PrP^{Sc} from human plasma by P1. A. Aliquots (900 µl) of human plasma obtained from two different individuals was spiked with 3 µl of 10 % total brain homogenate from hamsters infected with the 263K scrapie or from normal hamster in P1 was conjugated to streptavidin magnetic beads and were incubated at room temperature with (Lanes 1, 3, 4, and 6), followed by immunoblotting with 3F4. The conjugated beads were also incubated with 6 µl of 10 % total brain homogenate from wild type hamsters in two separate samples of human plasma (Lanes 2 and 5). Immunoblotting was performed using 3F4. B. A 10 % total brain homogenate from a hamster infected with the 263K strain of scrapie and brain homogenate from a wild type hamster were incubated in the absence (PK-) or presence (PK+) of 50 µg ml^{-1} PK for 1 h at 37 °C, followed by immunoblotting with 3F4.

DISCUSSION

We have discovered that the PrP^{Sc}-binding properties of the Kringle domains of plasminogen can be recapitulated in short, synthetic peptides based on the sequence of the Kringle domains. While the *in vivo* role, if any, of plasminogen in prion diseases is not clear, we have demonstrated that PrP^{Sc} binding properties of plasminogen may be exploited for diagnostic purposes, using short peptides based on the sequence of plasminogen's Kringle domains, and not the full length protein. In our studies, these peptides were able to bind to PrP^{Sc} from prion diseases from a variety of sources, with different etiologies, including BSE and vCJD. The peptides were found to bind to the core C-terminal fragment of PrP^{Sc}, PrP(27-30), as well as to the 6-7 kDa internal PrP fragment found in GSS. The internal fragment of GSS, migrating at 7-8 kDa spans residues 74-90 to 146-153(133-135) Because the peptides bind to PrP(27-30), the sequence of which is represented by recombinant PrP 90-231, it would appear that if the interaction is lysine based, as has previously been suggested for PrP interactions with plasminogen(127), that the N terminal lysine cluster (residues 23, 24 and 27) is not involved. The lysine cluster present at residues 101, 104, 106, and 110 remains in PrP 90-231, as well as in the internal fragment of GSS. However, the involvement of this cluster in binding is not clear because, in each peptide, 33% of the residues are positively charged. It seems unlikely that the lysine cluster is interacting with a peptide carrying an overall positive charge. Our results indicate that binding is retained when the positive charge of the peptide is present suggest that the lysine clusters may not be responsible for the binding to the peptides. The binding site for the peptides, and whether the interaction

with the peptides is direct, or through other elements in a complex with PrP^{Sc}, is a subject for further study.

PrP^{C} was not detected at any time using the peptide capture assay, even following prolonged exposure of the Western blots (data not shown). Previous work, as mentioned earlier, has revealed a binding site for the lysine binding region of the plasminogen Kringle domains in the lysine clusters of PrP^{C}, and PrP^{C} also contains a binding site for tPA. However, in our study, and in a similarly designed previous study using full length plasminogen, PrP^{C} was not detected in the brain homogenates of animal or human cases of prion disease(117, 118). Although there is a degree of proteolytic activity even in brain homogenate, this is not responsible for the absence of PrP^{C}, as it is clearly present in untreated brain homogenate of CJD unaffected individuals. It is unclear why recombinant PrP^{C} has been found to associate with plasminogen in vitro, but no studies using brain homogenate have found evidence of this binding. The physiological role of PrP is still poorly understood, so it is unknown whether the in vitro observations are physiologically relevant. There have been reports that the binding of PrP to plasminogen is a result of the detergent conditions of the experiment, and does not have any physiological role(136). Despite several suggestions, a direct in vivo interaction between plasminogen and PrP has never been proven. Therefore, a difference may exist between recombinant human PrP and the macromolecular environment in which PrP exists in the brain. Binding to other proteins, for example, could prevent the binding of plasminogen to PrP in this situation, even though such an interaction is theoretically possible.

The role of plasminogen in the disease course of scrapie has been investigated using mice infected with the RML strain, in an attempt to determine an in vivo role for

plasminogen in prion disease. As mentioned previously, no significant difference in overall survival between plasminogen knockout and wild type mice was observed. However, at the onset of symptoms, PrP^{Sc} accumulation was lower in the brains and spleens of plasminogen knock out mice, and corresponding differences in neuropathology were observed shortly after disease onset. These differences were also lacking at the terminal stage of the disease(130). Therefore, plasminogen was not believed to play an important role in the progression of prion disease, despite the finding of higher plasminogen concentrations in the brains of sCJD affected individuals in another study(128). A third study investigated a potential role for plasminogen in the cellular processing of the normal prion protein, and again, no difference in PrP^{C} cleavage was observed in plasminogen deficient mice(131). Despite the finding that plasmin was capable of cleaving PrP^{C} in vitro, this was not recapitulated in vivo. The lack of demonstrated in vivo interaction in both physiological and disease conditions enhances the usefulness of plasminogen-based peptides for prion disease diagnosis, as this suggests the interference of endogenous plasminogen binding in body fluids such as blood, is minimized.

Other peptide-based strategies have observed similar patterns in binding to PrP^{Sc}. The most popular strategy is to generate peptides based on the sequence of the prion protein itself, as the interaction between PrP^{C} and PrP^{Sc} is thought to be central in prion disease pathogenesis, while PrP^{C} undergoes only limited interaction with other PrP^{C} molecules(137-139). This technique has been successful with PrP-based peptides in an antibody scaffold, and as free peptides(137, 138). Although these peptides were derived from different sources than those in the present study, the characteristics of these peptides

were similar, and the positively charged residues were found to be important(138). This observation lends support to our data, and the combination of those observations with our own helps define the options for peptide based reagents for prion detection, as well as providing insights into the nature of PrP^{Sc}.

The apparent importance of charge in binding PrP^{Sc} may reflect structural features of the PrP^{Sc} molecule or aggregate. Although the structure of PrP^{Sc} has not yet been solved, various studies have provided insights into certain structural features. The structure has been probed using imaging based on uranyl acetate stained 2D crystals. Govaerts et al. hypothesized, based on results obtained from those studies, that a left handed β-sheet structure exposed negatively charged carbonyl groups along the protein backbone, which coordinated with the positively charged uranyl ion(140). Physiologically, this region may participate in the recruitment and conversion of PrP^{C} molecules through binding to its two positively charged domains(141). The importance of this region to PrP conversion has been demonstrated in a variety of studies. In the case of the peptides, it also serves as a potential region for direct interaction of PrP^{Sc}, as opposed to binding through another element in a complex. Furthermore, cationic interactions have been implicated in the pathogenesis of the most common fCJD mutation, E200K(142). An NMR structure of the mutant PrP^{C} revealed patches of positive electrostatic potential not present in the normal form, perhaps facilitating rapid recruitment by PrP^{Sc}(142).

With the continuing emergence of cases of both BSE and vCJD around the world, it is important that molecules that may show a preference for binding to PrP^{Sc} are investigated for diagnostic utility. There have been several reports of antibodies that

have been shown to bind to PrP^{Sc}, but not PrP^{C}, including the anti-DNA antibody OCD4, and an antibody raised against the YYR motif(85-89, 137, 143). The advantage of using short synthetic peptides is that they are cheaper and easier to produce than monoclonal antibodies, yet demonstrate a high degree of sensitivity to PrP^{Sc}. The ultimate goal of prion diagnostics is to develop a blood test detecting PrP^{Sc}. Such a test requires a high degree of sensitivity, as the concentration of PrP^{Sc} in blood is estimated to be extremely low. The buffy coat of whole blood, containing leukocytes and platelets, is estimated to have the highest concentration of PrP^{Sc} at 1 pg/mL during the symptomatic phase, and 0.1 pg/mL during the pre-symptomatic phase(94). In addition, there is a possibility that some of the PrP^{Sc} present in blood may be PK sensitive. Therefore, an ideal diagnostic reagent for PrP^{Sc} in the blood would not require the PK digestion step. The peptides show a high level of sensitivity in detecting PrP^{Sc}, and have been shown to capture PK sensitive PrP^{Sc}, so they provide a promising basis for the development of such a test. They also have a substantial advantage over full length plasminogen, an abundant plasma protein. Plasminogen undergoes many interactions with other blood proteins in vivo, as part of the fibrinolytic pathway, and these interactions may interfere with a blood based diagnostic test. This is less of a potential problem with the peptides, as they are synthetic, and have no natural interacting partners in the blood, potentially enabling a more specific reaction with PrP^{Sc}. Indeed, the peptides retained binding to PrP^{Sc} derived from hamster scrapie, spiked into human plasma. This demonstrates that endogenous plasma proteins do not significantly inhibit the prion-peptide interaction. In addition, the peptides are easily modified, so biotinylation can be performed for simple incorporation into capture assays, or other diagnostic assays, such as ELISA based methods. With reports of sensitive

prion-specific detection such as this, new avenues are opened for the earlier diagnosis, and possible treatment, of prion diseases.

CHAPTER 3

Aptamers Selected against PrP^{Sc} show High Specificity to Prions from Different Species

SUMMARY

Prion diseases are a group of fatal neurodegenerative diseases affecting both humans and animals. In recent years, these disorders have become a public health hazard due to the possibility of transmission from animals to humans, through the consumption of infected tissues. The pathogenesis of prion diseases involves a conformational change of the normal cellular prion protein (PrP^{C}) from an α-helical form to one rich in β-sheets, called PrP^{Sc}. PrP^{Sc} is insoluble in detergent, and is partially resistant to protease digestion. This latter property currently serves as the basis for differentiating PrP^{Sc} from PrP^{C} using antibody-based analyses. Using systematic evolution of ligands by exponential enrichment (SELEX), we generated a panel of DNA aptamers using the drowsy strain of hamster scrapie as a target. These aptamers show specificity for PrP^{Sc} derived not only from hamster scrapie, but also from prion diseases from other species and strains. Using a capture assay, the aptamers successfully bound to full length and PK treated PrP^{Sc} from the 263K strain of hamster scrapie, as well as from type 1 and type 2 sCJD, vCJD, mouse scrapie, sheep scrapie, and white-tail deer derived CWD. In addition, binding was observed in an electrophoretic mobility shift assay to buffy coat samples from scrapie afflicted sheep with 96% sensitivity and 100% specificity. Because binding was not observed to PrP^{Sc} from elk or mule deer CWD, or from BSE, these reagents are among the first specific for PrP^{Sc} to show species or strain specificity. This feature could potentially be exploited to develop aptamers specific for certain strains, serving as

invaluable tools in research, diagnostics, surveillance and potential treatment of prion diseases.

INTRODUCTION

There have been a variety of associations made between PrP and nucleic acids. Although the protein-only hypothesis dictates that there is no nucleic acid component associated with prion infectivity(2), there is some evidence that suggests nucleic acid molecules may have an accessory role in prion disease pathogenesis in vivo. In particular, it is suggested that host-encoded nucleic acids, especially RNA, may be required for efficient prion conversion in vitro(106). One study showed that single-stranded RNA, isolated from mammalian brain homogenate, is specifically required, with enzymes that degrade DNA, double stranded RNA or DNA/RNA hybrids do not affect the efficiency of in vitro conversion(106). In addition, RNA derived from invertebrate species had no effect on the conversion efficiency, suggesting that specific RNA may be required. Although it is possible to convert PrP^{C} to a PrP^{Sc}-like form in vitro in the absence of any cofactors, this process is inefficient, requiring the use of a 50-fold molar excess of purified PrP^{Sc}(144). This is an unlikely scenario in vivo, which suggests that accessory molecules are required. However, there is no direct proof that nucleic acids in particular act in this capacity in vivo(145). There is some suggestion that other endogenous accessory polyanions, such as glycosaminoglycans may be responsible, by acting as a scaffolds or surfaces for PrP^{C} recruitment by PrP^{Sc} seed molecules(146).

Although most studies concentrate on a potential role for RNA in prion conversion, DNA has also been implicated. One study, using recombinant murine prion protein, found that sequence-specific DNA binding converted the protein from an α-helical conformation to a β-sheet rich conformation, similar to that associated with PrP^{Sc}(147). In addition, double stranded DNA present in molar excess were capable of

inhibiting the aggregation of prion peptides, but that a high enough concentration of peptide aggregates can negate this effect. The authors propose that host derived nucleic acid may act as an intermediate, and catalyze the conversion of PrP^{C} to PrP^{Sc} if a threshold level of PrP^{Sc} is exceeded(147). A role for PrP as a nucleic acid binding protein has also been suggested based on the observation that it possesses nucleic acid binding and chaperoning properties similar to those of a human immunodeficiency virus (HIV) nucleocapsid protein(148). Regardless of whether the interaction between nucleic acids and the prion protein has any physiological importance, its potential application to prion diagnostics and therapeutics should be investigated.

Aptamers are artificial DNA or RNA oligonucleotide ligands specifically generated against particular non-nucleic acid targets, including proteins, drugs, amino acids or other molecules(149). They bind their targets with high affinity and specificity, with some aptamers demonstrated to possess affinity comparable to, or greater than, monoclonal antibodies, with K_d values in the picomolar range reported(150). Aptamers are generated using a technique called "Systematic Evolution of Ligands by EXponential enrichment" commonly referred to as SELEX(151). SELEX involves iterative cycles of selection and amplification, starting with a large pool of nucleic acids with randomized sequences. The randomized sequences are flanked by primer binding sites for the amplification step. Although there are a variety of different methods by which to achieve selection, the basic underlying method is to incubate the oligonucleotide library with the target of interest, followed by a step to partition bound from unbound materials(149). The selected nucleic acids, enriched for high affinity sequences, are amplified and the

selection is repeated. After several cycles, an enriched population of aptamers with high affinity for the target is available for characterization.

Aptamers have been selected against a variety of targets, including proteins, small molecules, carbohydrates, peptides and even whole cells(149). Some of the most striking studies have successfully selected aptamers against molecules with minor differences, such as aptamers capable of differentiating protein kinase C δ (PKCδ) from other isoforms. PCKδ has a high sequence homology with PKCθ, yet the isolated aptamers had significantly higher affinity for PKCδ. One potential obstacle with selection against proteins is the possibility of non-specific binding sites for nucleic acids(152). However, stringency of the SELEX typically increases with each successive round, so that low affinity, non-specific binding is likely to be largely eliminated.

There have been several previous attempts to generate aptamers, both DNA and RNA based, against PrP^{C} and PrP^{Sc} (153-160). Although these attempts have generated aptamers that efficiently bind PrP^{C} and PrP^{Sc}, no consensus sequence or sequence similarities have been observed. However, it should be noted that aptamer binding depends on conformation as well as sequence, so it is possible that certain structural elements may be responsible for PrP binding. Previous studies have demonstrated that a guanine-quadruplex structure is conserved among some RNA aptamers of different sequences(155). Although this structure is not widely reported in different studies, this may be because it is not predicted by routine determination of secondary structure. Some independently identified aptamers were found to have several sequence elements in common, including a shared 14 nucleotide sequence between aptamers generated by Mercey et al., and Rhie et al, and a shared 21 nucleotide sequence in another of the

Mercey aptamers with those identified by Proske et al(155, 156, 160). It is commonly predicted that PrP^{C} contains a nucleic acid binding site, which may be one element of its interaction with aptamers, in its N terminal region(155, 157). Two lysine clusters are located there, only one of which remains in PrP(90-231). Therefore, it appears that sequence independent interactions may occur at the N terminal of PrP^{C}, while binding sites in the C terminal region may be dependent on the sequence of the nucleic acid(154, 157). This is especially important in aptamers that bind either β-sheet rich recombinant PrP, or to PrP^{Sc} itself, as the C-terminal binding site identified in these studies is believed to be occluded in PrP^{C}, but exposed in PrP^{Sc} conformations, accounting for the PrP^{Sc} specificity.

Previous studies have only characterized aptamers against the same species and strain of prion protein against which they were selected. We have generated a panel of aptamers, selected against the drowsy strain of hamster scrapie, which show specificity for the 263K strain of hamster scrapie, sheep scrapie and human prion diseases, including types 1 and 2 sCJD and variant CJD. However, no binding was observed to chronic wasting disease-derived PrP^{Sc} (except for one aptamer with specificity for full length PrP^{Sc} from white-tail deer and elk CWD), or to that derived from bovine spongiform encephalopathy. This suggests that aptamers can potentially be used to differentiate PrP^{Sc} derived from different species and strains.

METHODS

Sodium phosphotungstate enrichment method: Extraction and protease K digestion of PrP^{Sc} from the brain tissue of scrapie infected hamster was conducted using sodium phosphotungstate enrichment method(24)**.** This enriched PrP^{Sc} fraction was used for the 9^{th} to 17^{th} round SELEX.

*Extraction of membrane protein from the buffy-coat***:** Briefly, 300 µl of buffy-coat sample collected from normal sheep or scrapie positive sheep (tested and supplied by Dr. Mark Hall, National Veterinary Services Laboratory, Ames, Iowa) was mixed with 1000 µl of ice-cold Buffer 1 [10 mM Hepes-KOH, pH 7.0, 10 mM KCl, 0.05 mM EGTA (Ethylene glycol-bis(2-aminoethylether)-N,N,N'N'-tetraacetic acid), and 0.05 mM dithiothreitol) supplemented with 1x Complete, EDTA-free protease inhibitors cocktail (Roche, Mannheim, Germany). The mixture was chilled on ice for 15 min. Then it was gently shaken on a tilt shaker for 60 min at 4ºC. The lysate was then centrifuged for 10 min at 3,500 x g at 4ºC. The supernatant was centrifuged for 2h at 100,000 x g. The pellet containing membrane fraction was resuspended in 200 µl of cold Buffer 2 (20 mM Hepes-KOH, pH7.0, 160 mM KCl, 0.1 mM dithiothreitol) supplemented with 1 x protease inhibitors cocktail. The suspended pellet was dissolved in Buffer 3 (20 mM Tris-HCl, pH 8.8, 1% Triton X-100) with gentle shaking on a tilt shaker overnight at 4ºC. To digest internal DNA, the suspension was then treated with 5 mM $CaCl_2$ and 1 µg/ µl Benzonase® Nuclease (EMD Biosciences, Darmstadt, Germany) for 2 h at 37 ºC with gentle agitation, 20 mM EDTA was used to stop DNase activity after digestion. The suspension was centrifuged for 30 min at 10, 000 x g. The supernatant was centrifuged

for 2 h at 286, 800 x g and the pellet was resuspended in cold Buffer 3. Proteinase K digestion was performed with 10 ng/µl Proteinse K (Promega Co., Madison, WI) for 1 h at 37 ºC with gentle agitation and then stopped by 1 x protease inhibitors cocktail and chilled on ice for 15 min. The suspension was centrifuged for 1 h at 286, 800 x g at 4ºC and the pellet was resuspended with cold Buffer 3 and stored at -80ºC.

Materials for SELEX: An aptamer library that consisted of a randomized 40-mer DNA sequence flanked by two 28-mer primer binding sites (5'-TTTGGTCCTTGTCTTATGTCCAGAATGC-N_{40}-ATTTCTCCTACTGGGATAGGTGGATTAT-3': where N_{40} represents 40 random nucleotides with equimolar A, C, G and T) was synthesized (Integrated DNA technology, Inc., Coralville, IA). The same manufacturer was used to synthesize all primers and aptamers applied in this study. The recombinant human PrP fragment consisting of amino acid residues 23-231 (rhuPrP 23-231, Abcam Inc., Cambridge, MA) and PrP^{C} (residue 23-231) extracted from normal hamster brain tissue served as the counter-selection target proteins in the first 8 rounds and 9-17 round of SELEX, respectively. PK treated hamster scrapie (Drowsy strain) served as the target proteins in 1-8 round of SELEX, the sodium phosphotungstate enriched PrP^{Sc} fragment (PK+) from Drowsy strain of scrapie hamster was used in rounds 9-17 of the SELEX procedure. A device for lateral flow chromatography (6 mm × 65 mm) consisting of a nitrocellulose (NC) membrane immobilized on a polymer support with aptamer releasing pad at one end and wicking pad at the other, was used as the solid phase support for SELEX procedures.

SELEX: Briefly, rhuPrP^{C} 23-231 and PrP^{Sc} were deposited as a thin line on the NC membrane and immobilized by air-drying. The NC membrane was blocked with 1%

bovine serum albumin (BSA) in phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBST). The aptamer library was diluted in PBST containing 1% BSA and applied on the releasing pad. After DNA molecules passed through rhuPrP23-231 (for rounds 1-8) or normal hamster PrP^{C} (PK-, for rounds 9-17), the spent library was exposed to PK treated PrP^{Sc} coated as a second line on the NC membrane, the solid phase was washed 6 times and each time with 50 µl high stringency-washing buffer. The PrP^{Sc} PK+ coated region of the NC membrane, where the high affinity aptamers were expected to bind, served as a template for PCR. Amplification was carried out with the forward primer, SK39 (5'-TTTGGTCCTTGTCTTATGTCCAGAATGC-3') and the reverse primer BioSK38 (5'- ATAATCCACCTATCCCAGTAGGAGAAAT-3'), which was biotinylated at the 5'end to enable removal of the reverse complement strand of the amplicon using streptavidin coated magnetic beads (Promega Co., Madison, WI) in order to restore the selected unbiotinylated strands of the original library for subsequent iterations of SELEX. Seventeen subsequent iterations of SELEX were performed independently against each molecule, respectively. Binding specificity and affinity of the 6^{th}, 9^{th}, 13^{th}, and 17^{th} aptamer pool were investigated by chemiluminescent EMSA analyses (LightShift Chemiluminescent EMSA Kit, PIERCE, Rockford, IL). In order to increase the specificity of binding, PK treated PrP^{Sc} from brain tissue of Drowsy scrapie hamster (sHPK+) and PrP^{C} from brain tissue of normal hamster (nHPK-), extracted and enriched using sodium phosphotungstate enrichment method, were serially diluted and loaded on the NC membrane as the targets in rounds 9-17.

Cloning and synthesis of the aptamer candidates: the candidates in the selected aptamer pool after the 12^{th} , 14^{th} and 17^{th} round of SELEX were cloned into TA vector (TOPO II,

Invitrogen Co., Carlsbad, CA) and 50 clones for each set of PrP^{Sc} molecules were sequenced. Based on the frequency of common sequences found among 50 clones and the theoretical secondary structures obtained using thermodynamics and mathematical modeling (http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/dna-form1.cgi), 36 sequences were obtained and evaluated for specificity and sensitivity test using gel shift approach. Twelve aptamers screened as above and 7 derivatized aptamers with nucleotide modifications from 7 original aptamers by deleting 1, 2 or 3 nucleotides were synthesized for further characterization.

*EMSA***:** Synthesized 5'-biotinylated aptamers without overhang fragments (0.25 ng) or heat-denatured amplicons (1.0-1.2 ng) of the SELEX aptamer pool (full length with over hang fragments), were purified using Qiagen MiniElute Purification kit and the concentration was determined using NanoDrop™ ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). They were incubated with PK digested or non-digested PrP^{C} and PrP^{Sc} 0.05 µg -3 µg (depending on the test) or Proteinse K digested membrane protein (4µg and 8µg) extracted from buffy-coat in the Binding Buffer (LightShift Chemiluminiscent EMSA Kit, PIERCE, Rockford, IL) added with 0.05 µg Poly(dA.dT)-Poly(dA.dT) (Amersham Bioscience, Piscataway, NJ) for 1 h at room temperature. The mixture was resolved by 0.5× Tris-Borate-EDTA (TBE) buffered native polyacrylamide gel (8%) electrophoresis. The 5'- end biotinylated aptamers or amplicons of the aptamer pools were transferred onto a positively charged nylon membrane (Schleicher & Schuell Inc., Keene, NH) and were visualized according to manufacturer's instructions for the PIERCE Lightshift Chemluminescence Kit.

Aptamer Capture Assay **:** 20 μg of biotinylated aptamers were conjugated to $2.3x10^8$ streptavidin superparamagnetic beads (Invitrogen, Carlsbad, CA) in 1 mL of 1X PBS at 37ºC for 20h. The conjugated beads were then incubated in 0.1% BSA in 1X PBS, at 37ºC for 4h, to block nonspecific binding. The capture assay was performed using 100 μL of the conjugated beads in 900 μL of capture buffer (1X PBS, 3% Tween 20, 3% Nonidet P-40), to which the 10% brain homogenate is added (5 μL of human brain homogenate, or 3 μL of animal brain homogenate). The mixture was incubated with constant rotation at room temperature for 3 h. Following the incubation, the beads were washed 3 times with Wash Buffer (1X PBS, 2% Tween-20, 2% Nonidet P-40), to remove unbound materials. The beads were then resuspended in SDS Sample Buffer (3% SDS, 2mM EDTA, 10% glycerol, 50mM Tris HCl, pH 6.8, 2.5% β-mercaptoethanol), heated to 100ºC for 10 minutes, and subjected to immunoblotting with 3F4.

Conformational Stability Immunoassay— Increasing concentrations of guanidine HCl (final concentration 0-3M) was added to 10% total brain homogenate of CJD (MM1). The samples were incubated for 1 hour at room temperature. A five fold volume of pre-chilled methanol was then added, for removal of the guanidine, followed by centrifugation for 30 minutes at 4°C. The supernatant was discarded, and the pellet resuspended in lysis buffer, with the aid of sonication. PK digestion, or an aptamer capture assay was then performed as described above.

RESULTS

DNA aptamers against hamster PrP^{Sc}. After six iterations of SELEX, gel shift assays indicated that the selected aptamer pool had higher affinity for PrP^{Sc} compared to the original aptamer library. Therefore, we have successfully generated a pool of aptamers with higher affinity for PrP^{Sc} than PrP^{C}. SELEX was continued for 17 additional iterations with decreasing amounts of the target protein, PK digested PrP^{Sc}. The aptamer pools generated from the 12th, 14th and 17th round were cloned, and 50 clones of each round were sequenced. Twelve aptamers were selected for further characterization, based on their redundancy in the libraries generated in each round, and inferred secondary structure using mfold (http://mfold.burnet.edu.au/dna_form) (Figure 1). Modifications to structures of DNA folding by sequence modifications were also simulated using mfold to select the best candidates for PrP^{Sc} binding studies.

Number	Aptamer ID	SELEX-enriched randomized sequences
1	A12-41	CCATGATGATGCCCCCGTGCGCAGGCTTTATAAACGCCCC
2	**A12-46**	**TCACCCACGCTACGAAGTAACAGGGATGGGGATAACACCG**
3	A12-46m	TC CCCACGCTACGAAGTAACAGGGATGGGGATAACACCG
4	A12-51	CAGGCCGGGAATGAATACACATCTATTGACCTGGTTAATA
5	A12-51m	CAGGCCGGGAATGAATACACATCTATTGACC GGTTAATA
6	A12-B2	TGAAGATATAGCTCCACGATTCGACCTCAGTACGGCGAGC
7	A12-B2m	TGAAGATATAGCTCCACGATTCG CCTCAGTACGGCGAGC
8	**A14-6**	**CAACCAAGGGAAGGGTAAGCGGAGGTACGGTCCGAATATA**
9	A14-24	CGGTAAGTGGTGATAATCAATCGACTCCCGAACTGCGGG
10	A14-27	GCGTGAGGGAATCCTAAATACGTGATTGTTATCCTGTCGT
11	A14-27m	GCGTGAGGGAATCCT CA CGTGATTGTTATCCTGTCGT
12	**A14-31**	**CAACGGGTAGGGATCAGCGCCAGTCCGTGATAAGGGGATA**
13	A14-31m	CAACGGGTAGGGATCAGCGCCAG CCGTGATAAGGGGATA
14	A17-46	TGCAGCAATAACCCTCCCTCGTCAGTTCGTCGGCCTGAGG
15	A17-46m	TGCAGCAATAACCCTCCCTCGTCAGTTCGTCGGCC GAGG
16	A17-50	CGTAGAGTGCTGCACGAACTGTATTAATCTCCTATCTACG
17	A17A-35	TAGACAGGAGTGCACACCGGATACGAACGATGCAGCCATA
18	**OA17p1-24**	**CTTATGTCCAGAATGCCAAACGAGACGGGGGGGACAATAG**
19	OA17p1-24mm	CTTATGTCC G TGCCAAACG GACGGGGGGGACAATAG

Figure 3.1 Sequencesof PrP^{Sc}-specific DNA aptamers. .Sequences of the randomized region of a selection of PrP^{Sc}-specific aptamers isolated using the SELEX technique. The sequences of the aptamers characterized in this study are highlighted in bold.

Aptamers bind to hamster scrapie PrP^{Sc} in a dose-dependent fashion. Aptamers 12A46, 14A6, 14A31 and 17OAp1-24 were conjugated, in quantities of either 2μg or 4 μg per reaction, and incubated with 3μl of 10%, 1% or 0.1% total brain homogenate derived from the 263K strain of hamster scrapie. With the 2 μg reactions, robust binding of PrP^{Sc} was observed to all aptamers, both with and without PK digestion (Figure 3.2A). Aptamer 17OAp1-24 also bound to small amounts of PrP^{C} from a hamster unaffected by scrapie. This cross-reactivity suggests the possibility of a sequence component to the binding. However, no PrP^{C} binding was observed with any of the other three aptamers. The binding to PrP^{C} was eliminated with the use of 1% homogenate (Figure 3.2B). Binding was retained to PrP^{Sc} by aptamers 12A46, 14A6, and 14A31, but not 17OAp1-24, again with and without PK digestion. Only one aptamer, 14A31, consistently retains binding to PK undigested PrP^{Sc} derived from 0.1% brain homogenate (Figure 3.2C). Therefore, this suggests that 14A31 has the highest affinity for the 263K strain of hamster scrapie, based on its ability to bind the lowest tested quantity of PrP^{Sc}, when no binding was consistently observed from the remaining aptamers. Overall, these results indicate that the aptamer capture of PrP^{Sc} is dose dependent, with more robust binding observed as the quantity of PrP^{Sc} in each reaction is increased. One aptamer, 17OAp1-24, binds PrP^{C} from 10% homogenate, but not 1% homogenate, suggesting that PrP^{Sc} binding is preferential, but that there may nevertheless be some affinity to PrP^{C} if it is present in sufficient quantity.

When the amount of biotinylated aptamer conjugated to streptavidin magnetic beads is increased to 4 μg, binding to 1% total brain homogenate derived from the 263K

strain of hamster scrapie was more robust than that observed in the 2 μg reactions (Figure 3.2D). Binding to PrP^{C} from uninfected hamsters was not observed in any of the aptamer reactions, despite the increase in aptamer quantity. Again, when 0.1% total homogenate was used, only 14A31 consistently captured the PK undigested form. The increase in quantity did not enhance the ability of any other aptamer to bind this small quantity of PrP^{Sc}; however, 14A31 capture of PrP^{Sc} was enhanced (Figure 3.2E). PrP^{Sc} binding to the aptamers, then, appears to be dependent on both the quantity of brain homogenate, as well as the quantity of the aptamers themselves. In general, the combination of 4 μg of aptamers per reaction and 1% brain homogenate appears to represent the most efficient combination for PrP^{Sc} detection. Robust binding is maintained, and the PrP^{C} binding present in the 2 μg reactions using 10% homogenate is not observed.

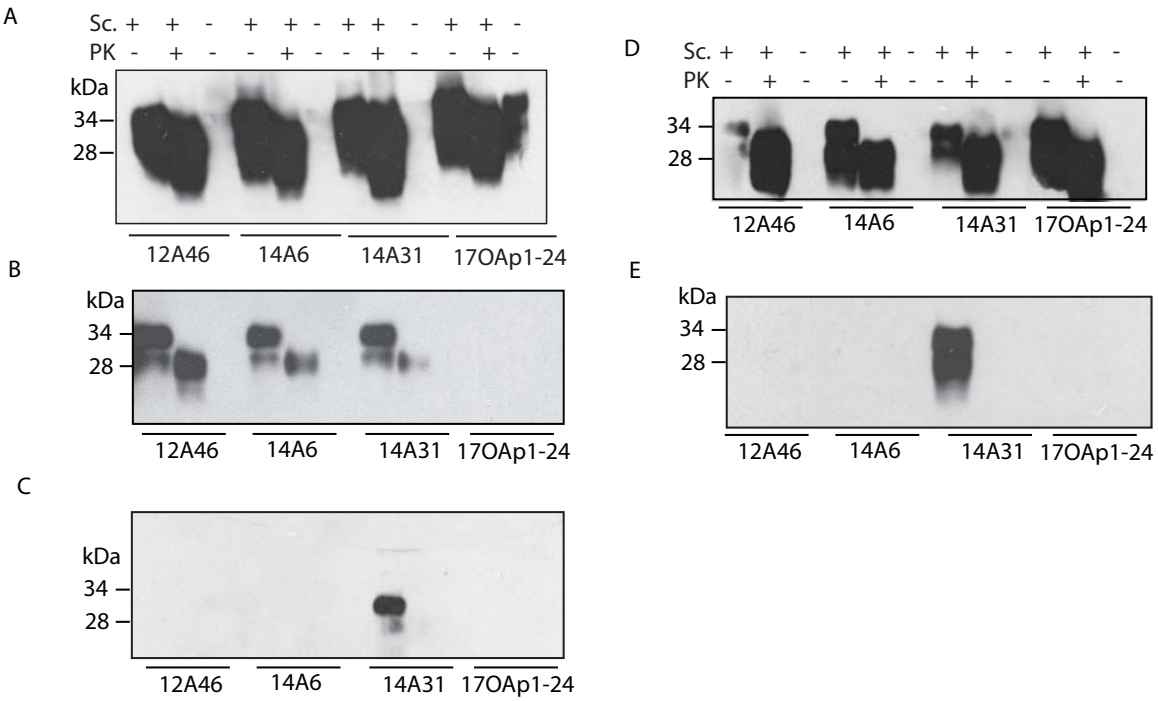


Figure 3.2 Dose-dependent aptamer capture of hamster scrapie A. 2μg of each aptamer per reaction, conjugated to streptavidin magnetic beads were used to capture PrP^{Sc} from 10% total brain homogenate from the 263K strain of hamster scrapie, with (lanes 2, 5, 8, 11) and without (1, 4, 7, 10) PK digestion, along with control samples from wild type hamsters (lanes 3, 6, 9, 12). Immunoblotting was performed using 3F4. B. Performed as described in A, using 1% total brain homogenate from the 263K strain of hamster scrapie. C. Performed as described in A, using 0.1% homogenate from the 263K strain of hamster scrapie. D. 4μg of each aptamer per reaction, conjugated to streptavidin magnetic beads were used to capture PrP^{Sc} from 1% total brain homogenate from the 263K strain of hamster scrapie, with (lanes 2, 5, 8, 11) and without (1, 4, 7, 10) PK digestion, along with control samples from wild type hamsters (lanes 3, 6, 9, 12). Immunoblotting was performed using 3F4. E. Performed as described in D, usi 0.1% total brain homogenate from the 263K strain of hamster scrapie.

The aptamers capture native PrP^{Sc} from two strains of human prion disease. 10% brain homogenate derived from type 1 and 2 sCJD, or from human tissue unaffected by CJD, was incubated with either 2 μg or 4 μg of each biotinylated aptamer conjugated to streptavidin magnetic beads, for 3 hours at room temperature, before immunoblotting with 3F4. In the 2 μg reactions, all four aptamers successfully captured PrP^{Sc} from both type 1 and type 2 sCJD, with no binding observed to PrP^{C} from CJD unaffected control tissue (Figure3.3A).

In the 4 μg reactions, all four aptamers captured PrPSc from both type 1 and type 2 sCJD in a greater quantity than the 2 μg reactions (Figure 3.3B). However, binding to PrP^{C} from control homogenate, unaffected by CJD, was also observed in all cases. As is the case with binding to hamster scrapie, the aptamers preferentially bind to PrP^{Sc}, with only a comparatively small amount of binding to PrP^{C} observed. Unlike the case of hamster scrapie, human PrP^{C} binding is only observed in the 4 μg reactions, and no aptamer appears to have a greater affinity for PrP^{C} than any other. This differs from hamster samples, in which case 17OAp1-24 has a greater affinity for PrP^{C} than 12A46, 14A6 and 14A31. As there are species-specific sequence differences between human and hamster prion proteins, this difference in PrP^{C} binding may be attributed to either these sequence differences, or to related differences in PrP^{C} conformation. Due to the highly conserved nature of the prion protein, hamster and human prion proteins have broadly similar structural characteristics; however there are local differences in the globular domains(17). Careful study of such differences can potentially provide clues as to the particular residues or structural motifs that constitute the binding sites of each aptamer.

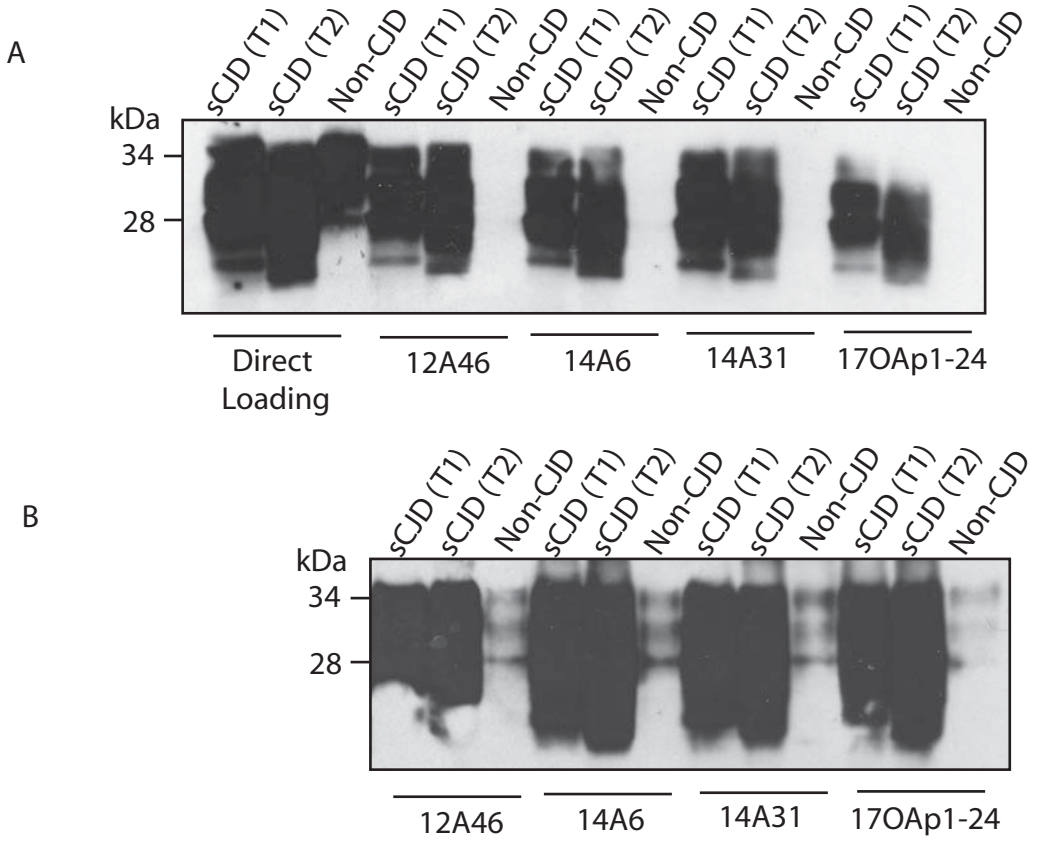


Figure 3.3 Aptamer capture of PrP^{Sc} from different subtypes of human prion disease. A. The aptamers, coupled to streptavidin magnetic beads at a quantity of 2 µg per reaction, were used to pull down PrP^{Sc} from 10% brain homogenate of individuals affected by type 1 sCJD (Lanes 4, 7, 10, 13), type 2 sCJD (lanes 5, 8, 11, 14) as well as CJD unaffected controls (lanes 6, 9, 12, 15), accompanied by direct loading controls from the same cases (lanes 1-3). B. Performed as described in A, using 4 µg of aptamers per reaction. Immunoblotting performed in all cases using 3F4.

Aptamer capture of three strains of PK digested human prion disease shows strain differences. Human prion diseases are known to occur in different strains, with subtle differences in conformation thought to be responsible for the differential migration on SDS-PAGE gel of PK digested type 1 and type 2 PrP^{Sc} in sCJD(37). Additionally, vCJD is believed to be a separate strain from sCJD, due to its origin from BSE. Even though its PrP^{Sc} gel migration pattern is approximately the same as type 2 sCJD, the predominance of each of the three glycosylation states differs, with the vCJD pattern having more in common with that observed in C-type BSE(30). The aptamer capture assay was performed, as described above, using PK digested 10% total brain homogenate from human patients affected by type 1 and 2 sCJD, as well as by vCJD. After incubation with 2 μg of aptamers conjugated to streptavidin magnetic beads at room temperature for 3 hours, immunoblotting with 3F4 revealed that, unlike with PK undigested PrP^{Sc} from CJD, there were differences in the affinity of each aptamer to the different strains of CJD (Figure 3.4A). Using computer-aided densitometry, a semi-quantitative analysis was performed on two separate immunoblots (Figure 3.4B). There were only small differences in the aptamer capture of type 1 PrP^{Sc} , while larger differences were observed in each aptamer's ability to capture type 2 PrP^{Sc} and PrP^{Sc} from vCJD. Overall, the strongest binding to PrP^{Sc} from type 2 sCJD and vCJD was observed with 12A46 and 14A31. 14A6 bound strongly to vCJD, but showed weaker binding to sCJD, especially type 2. Because there can be variations in the amount of PrP^{Sc} present in different sCJD cases, or even between brain regions in the same case, each capture assay was performed using homogenate derived from the same brain region and the same case of each type of

disease. This eliminates the possibility of variation in individual cases accounting for the observed differences in binding.

Although there were significant differences in aptamer affinity for PK digested PrP^{Sc} derived from human prion diseases in the 2 μg reactions, these differences were no longer apparent in the 4 μg reactions. When 10% brain homogenate from type 1 and type 2 sCJD, as well as vCJD, were incubated with 4 μg of aptamers conjugated to streptavidin magnetic beads for 3 hours at room temperature, strong binding to all tested strains of human prion disease was observed. Each aptamer captured the PK digested PrP^{Sc} in sufficient quantity to obscure the differences in affinity that were present in the lower quantity reactions. This indicates that the 4 μg reactions bind a greater quantity of PrP^{Sc} than the 2 μg reactions, such that discrimination in quantity is no longer possible with immunoblotting.

In these reactions, it is clear that the aptamers capture the C-terminal core fragment of PrP^{Sc}, commonly known as PrP(27-30). This region spans residues 82-231 in type 1 PrP^{Sc}, and 97-231 in type 2.

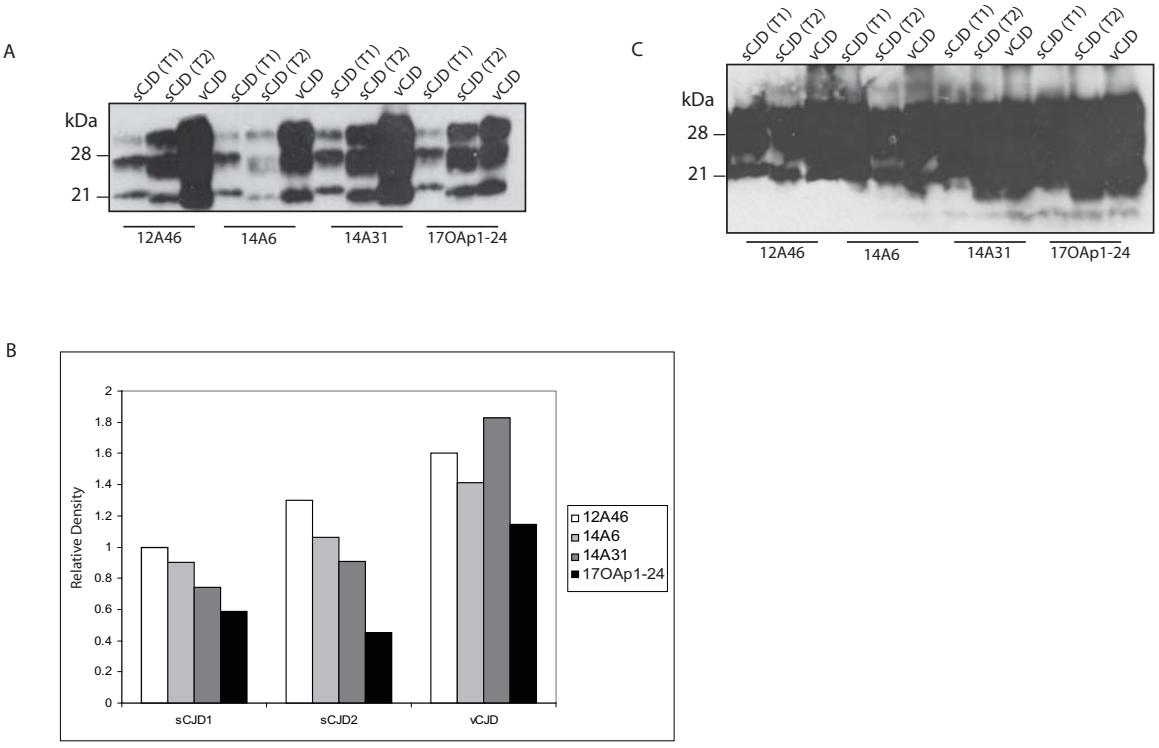


Figure 3.4 Aptamers capture of PK digested human PrP^{Sc} shows strain differences
A. Aptamers, conjugated to streptavidin magnetic beads at a quantity of 2 μg per reaction, were used to capture PrP^{Sc} from Proteinase K treated 10% brain homogenates of individuals affected by type 1 CJD (Lanes 1, 4, 7, 10), type 2 sCJD (lanes 2, 5, 8, 11) and vCJD (lanes 3, 6, 9, 12). Immunoblotting was performed using 3F4. B. Computer-assisted densiometric analysis of B, calculated using an average of two separate experiments. C. Performed as described in A, using 4 μg of aptamers per reaction.

Aptamers show differential binding to various animal species and strains of PrP^{Sc}. Using the aptamer capture assay, the aptamers were assessed for their ability to bind to PrP^{Sc} derived from a variety of animal species and strains. Biotinylated aptamers (2 μg per reaction) were conjugated to streptavidin magnetic beads and incubated with 10% total brain homogenate derived from the 22L, ME7 and RML strains of mouse scrapie, sheep scrapie, and chronic wasting disease from elk, white tail deer and mule deer (Figure 3.5). The results of the capture assay on the brain homogenates of scrapie-infected mouse (22L, RML, and ME8) and sheep scrapie show specific binding of the tested aptamers to the infected brain homogenates (Figure 3.5A). Aptamers clearly identified prions only in infected brain homogenates with or without PK digestion. The aptamers were more efficient in pulling down 22L compared with RML strain of mouse scrapie. The brain homogenates generated from normal sheep and scrapie-infected sheep were differentiated by the aptamers 12A46 and 14A31 with or without PK digestion (data not shown). While aptamer 17OAp1-24 detected CWD PrP isoforms from white tail deer brain homogenates, all aptamers failed to significantly bind to elk, or mule deer CWD derived PrP^{Sc}. All four aptamers failed to bind to PrP^{Sc} from 10% total brain homogenate derived from BSE, as well as experimental bovine prion diseases such as bovine CWD (data not shown). Taken together, these results suggest a potential role for sequence as well as structure in aptamer recognition, with differences in mouse, deer, elk and bovine prion protein sequence perhaps playing a role in aptamer binding.

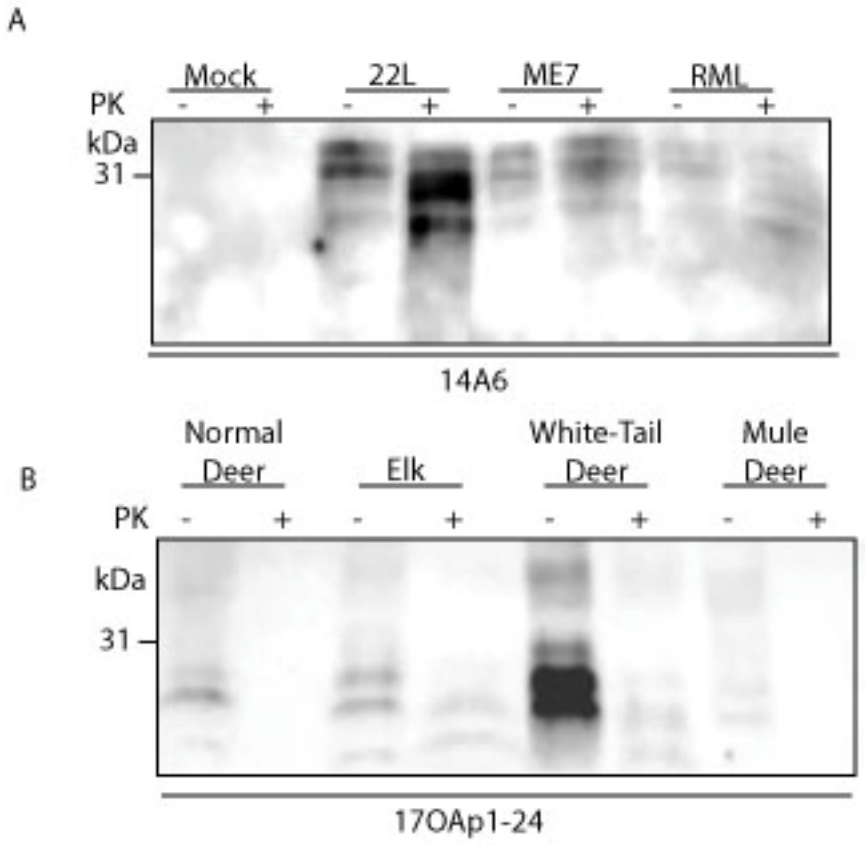
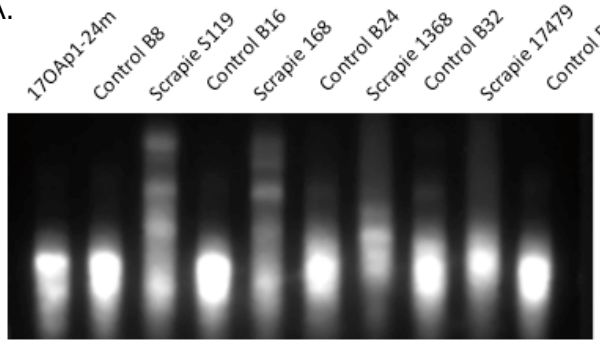


Figure 3.5 Capture of prions from multiple animal species show PrP^{Sc} specificity. A. Biotinylated aptamers were conjugated to streptavidin-magnetic beads and used in an aptamer capture assay to determine reactivity with three strains of mouse scrapie. 14A6 successfully captured PrPSc from the 22L, ME7 and RML strains. B. Aptamer 17OAp1-24 did not detect PrP in cases of chronic wasting disease in mule deer. Elk and white tailed deer PrP^{Sc} reacted with 17OAp1-24. Using 10% brain homogenate, there was some cross-reactivity with PrP^{C} derived from normal deer.

Aptamers differentiate buffy coats from scrapie and normal sheep To determine whether the selected aptamers have diagnostic potential in a blood-based assay, 26 buffy-coat samples collected from sheep with immunohistochemistry-confirmed scrapie and 16 control (Scrapie-negative) animals were processed for membrane protein extraction and prion detection using gel-shift analysis. As shown in Figure 3.6A, shifts were observed following 17OAp1-24 binding to proteins in buffy coats from scrapie infected sheep but not from unaffected animals. Of 26 IHC scrapie-positive samples, a shift was not observed in one case, and none of 16 control samples were positive in our EMSA leading to 100% specificity and 96.2% sensitivity and the agreement between aptamer based gel-shift and IHC assay is 97% (Figure 3.6B).

A.



B.

	Immunohistochemistry			
		+	-	
Aptamer Gel-Shift	+	25	0	**26**
	-	1	17	**17**
		26	**17**	**43**

Gel-Shift Sensitivity: 96.2%
Gel-Shift Specificity: 100%
Percent Agreement: 97.7%

Figure 3.6 Aptamer based gel shift analysis can detect prion infection in buffy coats of scrapie infected animals. Anticoagulated sheep blood samples from immunohistochemistry confirmed positive (samples S7, S81, S148, S361, and others labeled 'Scrapie") negative cases (B21, B22, B32, B168, and others labeled "Control") of scrapie were processed to separate buffy coats (white blood cell fraction). Buffy coat cells were lysed and total membrane extractions were performed to screen for the presence of prions using EMSA. Scrapie infected extracts were exclusively positive on gel shift analyses (Panel A). A total of 43 samples (26 scrapie confirmed and 17 negative) were analyzed by gel shift analysis, which showed a sensitivity of 96.2% and specificity of 100% (Panel B)

Aptamer binding to human PrP^{Sc} is both conformation and sequence dependent

Exposure of PrP^{Sc} to a denaturant such as guanidine HCl results in unfolding of the protein as the denaturant concentration increases, and this eventually renders it PK sensitive as the β-sheet rich conformation is lost. The rate of unfolding is characteristic of prion strains, with more stable structures remaining PK-resistant at higher concentrations of denaturant(24). Therefore, if aptamer binding depends on conformational features of PrP^{Sc}, rather than linear sequence, its affinity for PrP^{Sc} will be lost as the protein unfolds. Brain homogenate (10%) from sCJD (MM1) was exposed to increasing concentrations of guanidine HCl (0-3M), followed by methanol precipitation to remove the guanidine. After resuspension of the pellet, the brain homogenate was PK digested. At a concentration of 2.4M guanidine, PK resistance was lost (Figure 3.7A). A sample of the same homogenate, without PK digestion, was subjected to the aptamer capture assay using aptamer 12A46. While PrP was successfully captured, there was some, but not drastic decrease in binding as the guanidine concentration increased, suggesting that the affinity for PrP did not diminish with the loss of the PrP^{Sc} conformation (Figure 3.7B). Therefore, the binding may not be entirely conformation-dependent. PrP^{C} is known to contain a non-specific DNA binding site at its N terminus, consisting of two lysine-rich sequences. Although the major site of non-specific DNA binding was not present in the PK digested PrP^{Sc} target of the SELEX, it is possible that the aptamers are nevertheless capable of binding to it. As PrP^{Sc} unfolds, the exposure of this region is likely to be greater than is present in native PrP^{C}, allowing more efficient binding to denatured protein than to folded protein. Therefore, although there is a conformational preference to the aptamer binding, as demonstrated by its preferential

binding to PrP^{Sc} than PrP^{C}, there may also be a linear sequence element. A linear sequence component may also be the reason for observed differences in binding to the same prion strain within different species. CWD in different deer species is widely considered to be derived from the same prion strain(161, 162), yet aptamer 17OAp1-24 shows binding to only PrP^{Sc} derived from white-tail deer. Similarly, vCJD and BSE are also thought to be the same strain(30), but aptamer binding was observed to vCJD, but not BSE. Although there could be subtle, species-specific conformational differences that occurred as a result of adaptation to the new host, it may be possible that differences in binding are the result of sequence-dependence. PrP sequence differences between animal species are highlighted in Figure 3.8.

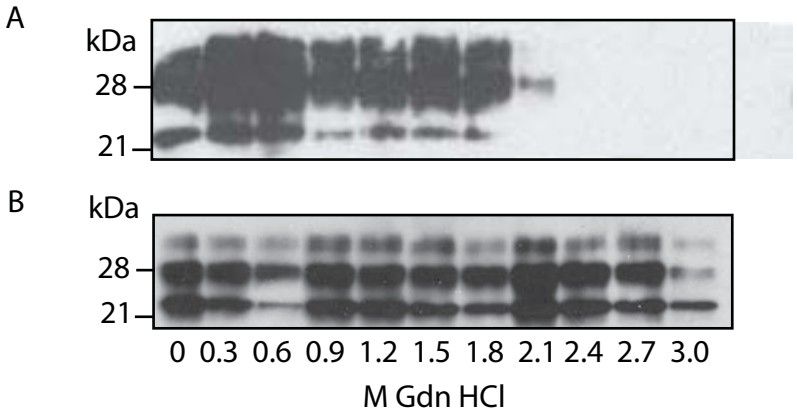


Figure 3.7. The binding of PrP^{Sc} to 12A46 is has both sequence and conformation components. A. Brain homogenate from sCJD (MM1) was incubated for 1 h with guanidine HCl (0-3 M), followed by digestion with 50 μg ml^{-1} PK for 1 h at 37 °C and immunoblotting with 3F4.. B. Brain homogenate from sCJD (MM1)was incubated for 1 h with guanidine HCl (0-3 M), followed by incubation with aptamer 12A46 conjugated beads and immunoblotting with 3F4.

```
elk MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQGSPGGNRYPPQGGGGW 60
wt  MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQGSPGGNRYPPQGGGGW 60
bov MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQGSPGGNRYPPQGGGGW 60
ham --MANLSYWLLALFVATWTDVGLCKKRPKPGG-WNTGGSRYPGQGSPGGNRYPPQGGGTW 57
      ****** * *    * *             *

elk GQPHGGGWGQPHGGGWGQPHGG--------GWGQPHGGGGWGQGG-THSQWNKPSKPKTN 111
wt  GQPHGGGWGQPHGGGWGQPHGG--------GWGQPHGGGGWGQGG-THSQWNKPSKPKTN 111
bov GQPHGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGGGWGQGG-THGQWNKPSKPKTN 119
ham GQPHGGGWGQPHGGGWGQPHGG--------GWGQPHGGG-WGQGGGTHNQWNKPNKPKTS 108
                         ********         *     *        *    *

elk MKHVAGAAAAGAVVGGLGGYMLGSAMSRPLIHFGNDYEDRYYRENMYRYPNQVYYRPVDQ 171
wt  MKHVAGAAAAGAVVGGLGGYMLGSAMSRPLIHFGNDYEDRYYRENMYRYPNQVYYRPVDQ 171
bov MKHVAGAAAAGAVVGGLGGYMLGSAMSRPLIHFGSDYEDRYYRENMHRYPNQVYYRPVDQ 179
ham MKHMAGAAAAGAVVGGLGGYMLGSAMSRPMLHFGNDWEDRYYRENMNRYPNQVYYRPVDQ 168
       *                           **   * *         *

elk YNNQNTFVHDCVNITVKQHTVTTTTKGENFTETDIKMMERVVEQMCITQYQRESEAYYQ- 230
wt  YNNQNTFVHDCVNITVKQHTVTTTTKGENFTETDIKMMERVVEQMCITQYQRESQAYYQ- 230
bov YSNQNNFVHDCVNITVKEHTVTTTTKGENFTETDIKMMERVVEQMCITQYQRESQAYYQ- 238
ham YNNQNNFVHDCVNITIKQHTVTTTTKGENFTETDVKMMERVVEQMCVTQYQKESQAYYDG 228
     *   *          * *                 *           *    *       *

elk RGASVILFSSPPVILLISFLIFLIVG 256
wt  RGASVILFSSPPVILLISFLIFLIVG 256
bov RGASVILFSSPPVILLISFLIFLIVG 264
ham RRSSAVLFSSPPVILLISFLIFLIVG 254
```

Figure 3.8 Sequence alignment of prion protein from four species. The sequences from the full length prion protein from elk (*Cervus elaphus nelsoni*), white-tail deer (wt; *Odocoileus virginianus*), bovine (bov; *Bos taurus*) and Syrian hamster (ham; *Mesocricetus auratus*) were aligned using ClustalW2 software (www.ebi.ac.uk/clustalw/). Sequence differences are indicated by asterisks.

DISCUSSION

We have isolated and characterized four DNA aptamers targeted against PrP^{Sc}. Although the target molecule for the generation of these aptamers was protease digested PrP^{Sc} derived from the drowsy strain of hamster scrapie, these aptamers showed affinity for prions derived from a variety of species and strains. This is not unexpected given that the prion protein sequence is highly conserved among species, and the conformation of the disease-associated protein is thought to be similar. Because aptamers have previously been demonstrated to have both sequence and structural elements that influence their binding, it is expected that the aptamers would have affinity to similar regions on different prion proteins. However, despite binding to prion proteins from two different strains of hamster scrapie, from types 1 and 2 sCJD, vCJD and sheep scrapie, no binding was observed to PrP^{Sc} derived from BSE and experimental bovine prion diseases (bovine CWD), or to that present in some CWD affected species.

The differential affinity of the aptamers for various species and strains of prion diseases represents one of the first reagents that have been reported with this characteristic. There have been a variety of published reports for PrP^{Sc}-specific reagents, including antibodies, proteins, peptides and other aptamers (85, 87, 89, 117, 118, 138, 139, 143, 153-160, 163, 164). In these cases, if binding to more than one species or strain of PrP^{Sc} was reported at all, there appeared to be little difference in affinity. For example, plasminogen was shown to bind CJD, BSE and sheep scrapie, with or without protease digestion, with approximately equal affinity(117). The same was true for the anti-DNA antibody OCD4(89). Although the binding was not quantified in either of these reports, robust binding was demonstrated by Western blot. Therefore, the

observation that the aptamers exhibit preferential binding to certain prion strains suggests that a more complex binding mechanism is involved in the aptamer interaction, compared to other reagents.

A variety of mechanisms have been proposed for PrP^{Sc} interaction with specific reagents. The antibodies 15B3 and YYR have been proposed to be conformational, meaning that their epitopes are exposed in the pathogenic conformation, but not in the normal, α-helix-rich conformation of PrP^{C}(85, 87). In this model, the epitope residues are not necessarily neighboring in the primary sequence, but may be accessible to the antibody in certain protein conformations, but not others. A similar mechanism is thought to underlie the binding of plasminogen and its related peptides, described in this work, as well as peptides based on the sequence of the prion protein. In the absence of a nucleic acid component, observed strain differences in prion diseases are thought to be enciphered in the conformation of the prion protein(33). It has been demonstrated previously that, for example, different strains of prion proteins have characteristic incubation times and neuropathological characteristics, as well as different in vitro conformational stability of PrP^{Sc}. These are accounted for by subtle differences in conformation of PrP^{Sc} from one strain to the next, perhaps influenced in part by sequence differences between species and polymorphic variations within species. While other reagents are likely to bind based on broadly similar characteristics of PrP^{Sc} structure, or even to accessory molecules, the aptamers we described appear to be able to differentiate the more subtle conformational differences of PrP^{Sc} between strains.

The possibility of a sequence component to aptamer binding cannot be eliminated. Indeed, a sequence component to the aptamer binding is a strong possibility in light of

observed binding to PrP^C under certain circumstances, as well as the binding observed to white tail deer CWD, but not to mule deer or to elk CWD. Chronic wasting disease is widely regarded to be a single strain of prion disease existing in several species, perhaps derived from cervid exposure to sheep scrapie(161, 162, 165). While transmissions of mule deer CWD to hamsters have yielded the possibility of two different strains within mule deer, it is possible that this strain divergence occurred within the hamsters themselves, as is suspected to be the case with transmissible mink encephalopathy HY and DY strains(165, 166). Therefore, the differential binding of the aptamers to various species affected by CWD suggests that sequence variation, rather than conformational variation, may account for this. Similarly, BSE and vCJD are also thought to be caused by the same strain, but the aptamers bind robustly to vCJD, without any binding observed to PrP^{Sc} derived from BSE. Again, it is possible that sequence differences between the human and bovine prion proteins may account for this observation. Although the conformations of PrP^{Sc} in the experimental bovine prion disease, bovine CWD is expected to be different from BSE, binding was not observed to PrP^{Sc} from either of these diseases, despite observed binding to sheep scrapie and its derivative, the 263K strain of hamster scrapie. This observation further implicates a role for prion protein sequence in aptamer binding.

Although sequence specificity can be important in the development of species-specific prion reagents, it presents a challenge in the development of reagents that lack cross-reactivity to PrP^C. In addition, certain species have highly polymorphic sites that might interfere with aptamer binding, resulting in a situation in which there may be differences in aptamer affinity for PrP^{Sc} derived from different individual animals.

Although these considerations were not incorporated in the present work, further refinement of the technique by which the aptamers are generated, including selection of the target protein and careful characterization of the aptamers within the pool. For example, PrP^C is known to bind non-specifically to nucleic acids, a process thought to be mediated through its N terminal lysine clusters(146). Therefore, to minimize this potential for non-specific PrP^C binding, the SELEX procedure must account for this. Because the focus of a diagnostic test is binding to the prion protein in near-physiological state, such as that thought to be present in peripheral body fluids, the SELEX procedure must incorporate full length, properly folded native PrP^C with the appropriate post translational modifications. This will ensure elimination of aptamers with affinity for any exposed sequences of PrP^C, its glycans, or a conformational interaction. In the present work, elimination of PrP^C binding has been largely successful. Although binding to PrP^C was observed in one of the aptamers tested, the PrP^{Sc} binding was strongly preferential, and in the case of human prion disease, was only observed when 4μg of aptamers was used in each individual reaction. The only case in which binding to PrP^C was demonstrated was with aptamer OA17pl-46, with 10% total brain homogenate from the 263K strain of hamster scrapie. Binding of hamster PrP^C to the other aptamers was not observed, and it was eliminated in OA17pl-46 with the use of 1% hamster brain homogenate.

The differences in binding of the aptamers between various species and strain illustrates that there may be important differences in sequence and conformation between strains which may be exploited to generate strain-specific aptamers. This is a challenging task, given the broad similarities between mammalian prion proteins, but it could

potentially be accomplished with careful, stringent selection, and the addition of negative selection steps for cross-reactive strains. Although RNA aptamers may be more suitable for this purpose, due to the conformational flexibility, previous characterization of DNA aptamers have indicated that the dissociation constants compare favorably with those of RNA aptamers(153). Nevertheless, the greater structural flexibility of RNA aptamers may be helpful in obtaining aptamers with greater specificity.

The BSE epidemic in the UK demonstrated for the first time the zoonotic potential of prion diseases. As evidence suggests that BSE and vCJD may be the same prion strain, a strain specific aptamer would be invaluable in detecting human prion disease derived from BSE or other animal prion disease exposure, even in cases where the clinical presentation is atypical. There is some evidence, based on transmission studies in transgenic mice, that suggests that if vCJD were to emerge among genotypes other than 129MM, the disease characteristics may differ(63). Even in humans, there have been reports of valine homozygous individuals diagnosed with an atypical version of sCJD, that may, in fact, be vCJD(64). An aptamer that bound exclusively to the vCJD prion strain would be able to confirm this speculative diagnosis, and also serve as a valuable tool in vCJD surveillance efforts.

Because CWD is endemic in many parts of North America, it is of greater concern for potential transmission to humans than BSE, of which there has only been a modest number of cases. Currently, no human case of prion disease has been connected to the consumption of CWD-infected tissue, and studies in transgenic mice have indicated that such transmission may be prevented by species barrier effects(67). Nevertheless, in light of the BSE crisis in the UK and Europe, it is prudent to remain

vigilant and maintain active surveillance. If CJD cases derived from CWD were to emerge, it would be a time and resource-intensive venture to confirm CWD origin. In addition, the phenotype of such a case is uncertain, and may not be quickly recognized. A strain-specific aptamer for CWD would greatly enhance CWD surveillance efforts by allowing the rapid identification of any emerging human disease.

We have demonstrated that aptamer 17OAp1-24 was able to distinguish buffy coats derived from the blood of scrapie-affected sheep from those of unaffected sheep, with 100% specificity and 96% sensitivity. This represents first time any PrP^{Sc} specific reagent has been shown to distinguish infected and uninfected animals using a blood-based assay. The buffy coat reactivity is very promising as an alternative testing platform for the blood-based diagnosis of prion diseases. Whether the interaction in buffy coat samples is directly with PrP^{Sc}, or with an associated molecule requires further characterization. Using the aptamer capture assay, we showed that all four aptamers tested were capable of binding PrP^{Sc} from vCJD, suggesting that the buffy coat EMSA may be adapted for vCJD detection. While the aptamers we characterized did not bind BSE, new aptamers could be specifically targeted to PrP^{Sc} derived from BSE, and applied to a similar assay. Although standardization of an EMSA is more challenging than an ELISA based method, further optimization and development of this assay has the potential for adaptation to slaughterhouse BSE surveillance, as well as to vCJD diagnostics.

In summary, we have selected and characterized aptamer candidates for binding to PrP^{Sc} with an aptamer capture assay to isolate PrP^{Sc} from brain homogenates. We also show the utility of selected aptamers in detecting PrP^{Sc} from membrane preparations of

buffy-coats of scrapie-infected sheep, suggesting a potential basis for a blood test. We show here that, 12A46, 14A614A31 and 17OAp1-24 captured three CJD subtypes. These same aptamers captured PK-digested PrP from scrapie-infected sheep, mouse (3 different strains), with or without PK digestion, but did not capture PrP from normal animals, with the exception of 17OAp1-24, which captured PrP^{C} from wild type hamster brain homogenate. These data demonstrate that selected aptamers bind selectively to PrP^{Sc} even in the absence of PK treatment and that some aptamers are specific to prions from a wide variety of species, while others have greater affinity for certain strains. Aptamers such as these have a great potential to open new avenues into prion disease research, diagnostics and treatment.

CHAPTER 4

SUMMARY AND CONCLUSIONS

4.1 General Discussion

With the onset of the BSE epidemic in the UK in the 1980s and 1990s, and with the subsequent discovery of vCJD in humans, prion diseases present an emerging public health problem. In the UK alone, there were more than 185000 confirmed cases of BSE, and epidemiological estimates have placed the total number of cases into the millions(167). Therefore, there was widespread exposure of the population to BSE-contaminated beef. So far, as of September 2008, there have been 206 vCJD-related deaths worldwide, and there are 5 suspected cases still living in the UK, Portugal and Saudi Arabia. All confirmed cases so far have appeared in codon 129 methionine homozygotes. As cases of vCJD are on the decline, with only a handful of cases diagnosed in recent years, it may appear as though the threat has passed. However, there are several factors that illustrate the need for continued vigilance. The first is the demonstration that, unlike other human prion diseases, vCJD can be transmitted by blood transfusion(60). Furthermore, in one of the transfusion cases, the affected individual was heterozygous at codon 129, the first time evidence of disease had been discovered in this genotype(61). However, the individual showed no clinical signs of disease, and had died of a different cause. For the first time, this raised the possibility of asymptomatic carriers of vCJD in the population, who could unwittingly expose other individuals through blood or organ donations, or even through contaminated surgical instruments, as has previously occurred with the iatrogenic transmission of sCJD.

A second factor in favor of continued surveillance of prion diseases is the demonstration in transgenic mice that vCJD can infect all genotypes(63). While it is difficult to infect mice expressing the human prion protein with BSE, vCJD was readily passaged between humanized methionine homozygotes. Furthermore, heterozygotic mice and valine homozygotes were also infected, although with a lower attack rate and longer incubation times. The disease phenotype also differed among these groups, raising the interesting possibility that vCJD in other genotypes may differ from the classic presentation, and potentially remain unrecognized(63). This scenario in transgenic mice approximates what occurred with the transmission of kuru(55). Those initially infected with kuru were also homozygous for methionine. As time passed, additional cases emerged among other genotypes, with incubation periods years, or even decades long(55). While vCJD has not yet been observed to follow this pattern, there is no reason to assume that there is no further threat.

Continued surveillance of prion diseases is also vital in the agricultural industry, in order to prevent prion infection in livestock and infected animals from reaching the human food chain. Current diagnostic assays for prion diseases require brain tissue samples, obtainable only at autopsy or during risky biopsy procedures(72). Such techniques are impractical and inadequate for widespread screening of either potentially exposed humans or of animals destined for human consumption. Therefore, there is an obvious need for the development of highly sensitive reagents that can be adapted to high throughput screening, ideally of blood or another peripheral body fluid. Because the primary sequence of PrP^{C} and PrP^{Sc} are the same, it is difficult to generate antibodies against PrP^{Sc} without cross reactivity to PrP^{C}. Nevertheless, several have been described

in recent years, most of them with conformational epitopes(85, 87-89, 143). This means that the residues that the antibodies recognize are either only exposed in the disease-associated conformation, or are only in close enough proximity to facilitate antibody recognition in the disease-associated conformation. Conformational reagents have a particular advantage in the detection of PrP^{Sc} because structural features comprise the key difference between PrP^{C} and PrP^{Sc}.

4.2 The Kringle-Based Peptides

Our first approach to developing a PrP^{Sc}-specific reagent was based on the observation that the kringle domains of plasminogen bound to PrP^{Sc} from different species, including that associated with human and bovine prion diseases. Using residues that appear frequently throughout the first three kringle domains, and especially those surrounding the disulfide bonds, we generated short peptides, 12-15 residues in length, that were able to detect PrP^{Sc}, but not PrP^{C}. The sequences of these two peptides are similar in that they are made up of repetitive sequences of arginine or lysine, tyrosine and glycine. We have shown that these peptides are capable of binding to PrP^{Sc} from a variety of species and strains, including chronic wasting disease, bovine spongiform encephalopathy, and a variety of forms of CJD, including type 1 and type 2 sporadic, genetic and variant. The binding was robust despite known sequence differences and purported strain-specific conformational differences.

Because of the specificity to PrP^{Sc} from different sources, the peptides were tested for conformation dependent binding using a guanidine HCl assay(168). As PrP^{Sc} denatures with increasing concentrations of guanidine, it becomes increasingly protease

sensitive as its conformation is lost. As the native conformation of PrP^{Sc} is lost, the affinity of peptide 1 decreases as observed in a Western blot. This suggests that the binding of the peptides to PrP^{Sc} is dependent on protein conformation, not sequence. In addition, a small amount of binding was observed to PrP at guanidine concentrations at which the protein is fully protease sensitive, indicating that if PrP^{Sc} is bound, it is the PK sensitive form. Previous studies have demonstrated that PK sensitive PrP^{Sc} makes up the majority of PrP^{Sc} present in human prion diseases(26), so the ability to recognize this form is a valuable asset to a diagnostic assay. A second interesting feature of the PrP^{Sc} binding peptides is their dependence of positively charged residues for binding. If peptides lacking the positively charge arginine residues are synthesized, no binding to PrP^{Sc} or to PrP^{C} is observed. Elimination of the tyrosine residues does not affect binding, and substitution of the arginine with a negatively charge aspartic acid residue does not restore binding. This property has been observed previously in assays designed for the detection of PrP^{Sc} and may provide valuable structural insights into PrP^{Sc}(138).

4.3 The DNA Aptamers

The aptamers represented our second strategy for the development of PrP^{Sc}-specific reagents. Aptamers are short oligonucleotides that can be generated using the well-established SELEX procedure against a target molecule of interest(151). The SELEX procedure we used incorporated a negative selection step, in which recombinant PrP^{C} was immobilized to a nitrocellulose membrane strip such that the aptamer library would pass through PrP^{C} before reaching the immobilized PrP^{Sc}. By eliminating aptamers with high affinity for PrP^{C} from the pool that bound PrP^{Sc}, the potential for cross reactivity to PrP^{C}

is reduced. The aptamer panel we characterized was generated using the drowsy strain of hamster scrapie. Using an aptamer capture assay, the aptamers captured PrP^{Sc} from total brain homogenate of the 263K strain of hamster scrapie, as well as from human cases of sCJD containing type 1 and type 2 PrP^{Sc}, as well as vCJD. In all of these cases, both protease digested and undigested forms were captured. However, the aptamers seem to discriminate other prion strains, as they failed to capture PrP^{Sc} from mule deer CWD, but one aptamer successfully captured PrP^{Sc} from white tail deer CWD and Elk. In addition, the aptamers failed to capture PrP^{Sc} from BSE, or from other experimental bovine prion diseases.

When binding to PK digested PrP^{Sc} from human prion diseases, the aptamers did not bind to it with equal affinity. When an average of two experiments was calculated based on results obtained from densiometric analysis, there were differences in how much PrP^{Sc} in each subtype was captured by each aptamer. Due to differences in the quantity of PrP^{Sc} in each case, only binding of each aptamer to the same case was compared. Overall, 17OAp1-24 bound most weakly to all tested forms of human prion disease. The best binding to types 1 and 2 sCJD was observed with aptamer 12A46, although there was not a large difference relative to the other aptamers. 14A31 bound the strongest to vCJD, but relatively weakly to sCJD. While none of the aptamers were entirely strain-specific, this differential binding of different strains from the same species indicates that conformation likely plays a role in the binding. Although there are polymorphic sites located within the human prion protein, the resulting sequence differences would be minimal.

The features of PrP^{Sc} binding to the aptamers were dependent on both the amount of PrP^{Sc} in the reaction, with stronger binding observed as the quantity increased, but also on the amount of aptamer used in each reaction. When 4 μg of aptamers were used to capture PK digested PrP^{Sc} from human prion diseases, there was no obvious difference in the amount captured.

4.4 Similarities and Differences

The aptamers and the peptides have several features in common. Most importantly, they bind to PrP^{Sc} largely in the absence of PrP^{C} binding, from a variety of species and strains. The peptides captured all tested species and strains, and the aptamers were observed to bind PrP^{Sc} from hamster and humans, despite sequence and strain differences between the associated prions. With few exceptions, binding was observed to both PK digested and full length PrP^{Sc}, in the absence of binding to PrP^{C}. This indicates that the binding may largely depend on the conformation of the prion protein. However, if prion strains, even in different species are conformationally similar, this suggests that perhaps conformation alone is not solely responsible for binding to the aptamers. Studies of the transmission characteristics and protein biology of vCJD and BSE have concluded that these arise from the same prion strain(30, 39). However, despite binding to vCJD, no aptamer binding was observed to BSE, suggesting that perhaps the interaction depends on exposed residues that are present in human PrP^{Sc}, but not bovine. Due to the highly conserved nature of the protein, this narrows potential interaction sites to only a few possibilities. No detailed structural information is available for PrP^{Sc}; however, PrP^{C} structure has been solved for a variety of species, so it is possible to determine the

relative positions of residues in question, whether they are present on the surface, or buried within the molecule. Since spatial arrangement, not just exposure on the surface, may also be important, it is not likely possible to determine an exact interaction site with PrP^{Sc} without more structural information.

The main difference between the peptides and the aptamers is that the aptamers demonstrated species and strain differences in their ability to capture PrP^{Sc}, whereas the peptides appeared to capture all tested species and strains with approximately equal affinity. In addition, binding to PrP^{C} was observed with the aptamers under certain conditions, described previously, and one aptamer in particular, 17OAp1-24, bound to PrP^{C} derived from 10% brain homogenate from the 263K strain of hamster scrapie. The aptamers showed a significant decrease in binding to hamster PrP^{Sc} when the quantity was reduced 10-fold to 1% brain homogenate, whereas peptide 1 continued to bind robustly to 0.5µL of 10% homogenate, which was close to a 10-fold reduction. While this reduction was overcome by increasing the aptamer quantity in each reaction, other experiments show that significant reduction in peptide quantity still results in strong binding (data not shown). This suggests that the peptides may bind PrP^{Sc} more efficiently than the aptamers, although this has yet to be quantified.

The basis for binding in both the aptamers and the peptides is unclear. Interestingly, the lysine clusters located in the N-terminus of the prion protein have been implicated in both the binding of nucleic acids, and that of plasminogen. The first lysine cluster is known to bind nucleic acids non-specifically, and could well play a role in certain aspects of aptamer binding. However, the N-terminal lysine cluster, located at residues 25-34 is cleaved after PK digestion, leaving only the second lysine cluster at

residues 101-110(37). Previous studies of aptamer binding to PrP^C have indicated that the primary interaction site is located at the first lysine cluster, and that aptamers with a preference for the β sheet conformation interact more C-terminally, perhaps interacting with residues that are better exposed in that conformation(155, 157, 159, 160). The C-terminal binding is consistent with observed binding to PK digested PrP^{Sc} in this study. Binding to the first lysine cluster may be non-specific, and could potentially account for the low background binding to PrP^C observed when large quantities of aptamer are used.

In vitro interaction between plasminogen and PrP^C has been well characterized, as discussed in Chapter 2. Mutagenesis studies determined that both lysine clusters are required for the interaction(127). However, it is unlikely that peptide binding to PrP^{Sc} depends on these lysine clusters, again because the N terminal lysine cluster is eliminated by PK digestion. The mutation of this lysine cluster in PrP^C abrogates binding to plasminogen, but binding of PrP^{Sc} to the peptides remains unaffected. Furthermore, the interaction between the peptides and PrP^{Sc} appears to depend on positively charged residues. It seems unlikely that the arginine residues in peptide 1 would be interacting with the lysine cluster in PrP, as this would be subject to unfavorable charge interactions.

Direct interaction between the prion protein and the peptides or aptamers has not been proven. It is not known which molecules, if any, associate with PrP^{Sc} deposits in vivo, or which molecules are associated with it as artifacts of the homogenization process or as a result of buffer chemistry. Although the protein-only hypothesis states that no nucleic acid is required for the conformational change, nucleic acids of varying lengths have co-purified with PrP^{Sc}(145). These are thought to be host encoded, and not relevant to the pathogenesis of the disease; however, this serves to illustrate the potential for

accessory molecules to be present. The binding characteristics of the peptides especially raise questions about whether direct interaction is occurring. Binding is observed to the PK digested for of human type 1 and type 2 PrP^{Sc}, as well as to the internal fragment found in GSS(135). Because there are no overlapping sequences in these fragments, this suggests that either the peptides are not limited to a particular sequence, but instead bind to particular sequence characteristics, or that they bind to an accessory molecule associated with PrP^{Sc}.

The ultimate goal of prion diagnostics is to develop a minimally invasive, highly sensitive test to facilitate large scale, pre-symptomatic screening of populations of animals, or humans with a history of prion exposure. There are some promising technologies emerging, such as PMCA, which can aid in the detection of minute quantities of PrP^{Sc}. However, since it has been reported that PMCA can generate infectious prions *de novo*, its use as a diagnostic tool faces serious challenges(108). The direct detection of PrP^{Sc} in the peripheral body fluids of terminal animals has not yet been accomplished, so pre-symptomatic detection remains a formidable challenge. It has been reported that the majority of PrP^{Sc} in the brains of CJD patients is PK sensitive, and the same is expected to be true within the peripheral fluids. Therefore, diagnostic reagents aimed at detecting PrP^{Sc} under these conditions must be able to differentiate the two forms of the protein in the absence of PK digestion. Because of this requirement, there has been much investigation into conformational reagents, including the mini-kringle peptides and the DNA aptamers we have identified in this work. Although the principle of differentiating two conformations of the same protein is well-established, in order to be practical as a diagnostic tools, further study is required on enhancing the sensitivity of

the test to appropriate levels. The concentration of PrP^{Sc} in the blood is predicted to be extremely low, with 1mL of hamster blood thought to contain 0.1-1 pg PrP^{Sc}, so sensitivity is a key issue(107). Some recently developed platforms, such as the DELFIA-based conformational-dependent immunoassay are approaching these levels of sensitivity(24). It is possible that this, or a similar technique, combined with enrichment of PrP^{Sc} using a reagent such as the aptamers or peptides, may lead to a sufficiently sensitive detection platform for blood borne PrP^{Sc}.

In summary, we have characterized two separate groups of reagents that preferentially bind to disease-associated prion protein. The first is a group of peptides based on the kringle domains of plasminogen, and the second are DNA aptamers generated using the SELEX procedure, with the drowsy strain of hamster scrapie as a target. They both successfully captured PrP^{Sc} from a variety of species and strains, although the affinity of the aptamers was primarily limited to human and hamster PrP^{Sc}. PrP^{C} binding was generally not observed, indicating that the interaction of these reagents with the prion protein depends on protein conformation. Elimination of the protease digestion requirement of current prion diagnosis could increases the assay sensitivity substantially, as it is believed that PK sensitive PrP^{Sc} may be more prevalent, even in the brain, than the PK resistant form. In particular, the peptides can bind to PrP^{Sc} even after it had been rendered PK sensitive by guanidine denaturation. In addition, the PrP^{Sc} in blood is believed to be PK sensitive, so a blood test would rely on a reagent that is able to detect that form, but not PrP^{C}. The peptides successfully captured spiked PrP^{Sc} in plasma, indicating that any endogenous factors in plasma do not inhibit the interaction. The aptamers successfully differentiated sheep scrapie afflicted from unaffected buffy

coats derived from sheep blood. Therefore, due to their ability to differentiate the disease-associated prion protein from its cellular counterpart in the absence of PK digestion, and the promising results from the blood-based assays, these reagents can potentially be adapted to a diagnostic platform for prion diseases.

4.5 Future Directions

The observed robust binding of the peptides to PrP^{Sc} from a variety of species and strains represents a promising direction for the development of a diagnostic test for prion diseases. A particular advantage of the use of peptides in this fashion is their demonstrated ability to bind PrP^{Sc} with or without protease digestion, in the absence of PrP^{C} binding. The sensitivity of the individual peptides remains unquantified, so the first step in the development of a peptide-based diagnostic test is to perform a quantitative comparison of P1 and P2. This requires the adaptation of the peptide capture assay to an ELISA based format, in combination with a PrP-specific antibody. The most significant challenge in the development of this platform is the demonstrated inability of many of the most common anti-PrP antibodies, such as 3F4, to efficiently bind to native PrP^{Sc}(89). In order to maximize the sensitivity of an ELISA-based test, a variety of anti-PrP antibodies will have to be tested. Previous attempts to design ELISAs for PrP^{Sc} have been successful with some antibodies(169, 170). Because the structure of native PrP^{Sc} remains unsolved, it is not known which residues are exposed and accessible to antibodies. The majority of the conformational change is thought to center around helix 1, comprising residues 144-156(171, 172). Therefore, antibodies that efficiently bind PrP^{Sc} may require epitopes in regions unaffected by conformational change and subsequent aggregation, or epitopes that are comprised of residues that become exposed as a result of the conformational change.

Once a suitable ELISA-based platform has been established, the peptides can be compared in order to determine which of the two is most suited for a diagnostic test of optimal sensitivity. It is especially important to determine sensitivity and specificity for

prion diseases of most importance to public health: bovine spongiform encephalopathy, variant Creutzfeldt-Jakob disease and chronic wasting disease. Decreasing quantities of homogenate from each type of prion disease can be used to establish the minimum level of detection of each peptide/antibody combination. In addition, varying quantities of homogenates both affected and unaffected by prion disease should be blindly tested in order to determine sensitivity and specificity. Since a blood test is the most desirable type of diagnostic test for prion disease, additional plasma spiking experiments can be performed in order to determine whether plasma alters the test sensitivity.

Because the quantity of PrP^{Sc} in the blood is thought to be significantly reduced compared to brain tissue, it is likely that a peptide ELISA alone is not of sufficient sensitivity to detect blood-based prions. Therefore, adaptation of this method to further enhance sensitivity is required. This may be achieved by enrichment of the PrP^{Sc} within a sample, and by selection of the appropriate detection system. The sensitivity of the conformation-dependent immunoassay was substantially improved by the use of sodium phosphotungstate precipitation, which leads to the generation of a PrP^{Sc} enriched pellet(24). Under optimal conditions, including protease digestion, this can lead to PrP^{Sc} purity of greater than 90%(24). However, as mentioned previously, if the PrP^{Sc} present in blood and the majority of PrP^{Sc} present in brain is protease sensitive, the protease digestion step should be avoided. Nevertheless, PTA precipitation enriches PrP^{Sc} even in the absence of protease digestion, and is an important first step in optimizing the sensitivity of PrP^{Sc} detection in samples containing minute quantities.

The method of detection is perhaps the most important aspect of sensitivity optimization for a peptide-based assay. It is possible that the sensitivity of the peptide

capture assay in its present form is limited by the use of a Western blot for prion detection, and this is also likely to be true for a standard horseradish peroxidase-based ELISA. Several ultra-sensitive detection platforms have been developed in recent years, many of them based on the ELISA. Among them are fluorescence-based detection systems such as the DELFIA (dissociation-enhanced lanthanide fluorescent immunoassay), which offer superior sensitivity, and in previously developed prion assays, sensitivities of 5 pg/mL or less have been reported.

While the above methods of development of an ultrasensitive prion detection platform can also be applied to the aptamers, this fails to take advantage of one of their most interesting characteristics. The strain preference demonstrated by some of the aptamers represents an interesting direction for future study. Despite the aptamers having been generated against the drowsy strain of hamster scrapie, strain preferences were observed in human prion disease as well as in chronic wasting disease and in natural and experimental prion diseases. While none of the current aptamers are entirely strain-specific, development of such an aptamer is possible. This type of strain specificity is especially important to prion diseases that may be transmitted from animals, such as BSE. The aptamers described here can distinguish BSE from vCJD, even though these are widely considered to be the same strain. This could perhaps be due to sequence differences between the human and bovine prion proteins, as mentioned previously. In order to focus on strain specificity, SELEX can be adapted to isolate aptamers against the prion species or strain of choice. For example, if an aptamer is desired that can differentiate variant from sporadic CJD, the negative selection SELEX can be performed using PrP^{Sc} from sporadic and variant CJD as a target. The isolation of PrP^{Sc} is critical in

the SELEX procedure, to avoid inadvertent selection of aptamers targeting factors that may non-specifically associate with PrP^{Sc}, either in vivo or as artifacts in the homogenization process. While the SELEX procedure performed as a part of this work employed PTA precipitation in combination with protease digestion as the isolation method, to enhance strain-specific selection, highly purified fractions using a more extensive purification procedure can be used. Purity is usually determined using a combination of silver staining and Western blot. There are several proteins, and even some polynucleotides, which are known to co-purify with PrP^{Sc}, so purity may not be optimal. However, it is possible that some of these accessory molecules may serve to differentiate PrP^{C} from PrP^{Sc}, so extremely high levels of purity may not be necessary.

The key issue in the development of strain specific aptamers is the characterization of aptamers with affinity for PrP^{Sc}. Initially, cross-reactivity between species and strains, and even with PrP^{C} is likely. Absolute strain specificity may not even be possible, based on the similarities in prion sequence and structure, as well as potential non-specific binding between proteins and nucleic acids. It may be more practical to develop aptamers that can differentiate one strain from another, such as sCJD from vCJD, or BSE from CWD, rather than attempt to create an entirely strain specific aptamer. This can be accomplished using the same negative selection principles described above, with initial rounds eliminating most PrP^{C} binding, and further rounds with the enriched pools attempting to differentiate the strains of interest. Again, careful characterization is required to ensure that cross reactivity does not occur in a capture assay format. Successful generation of aptamers that can differentiate the BSE/vCJD strain or even the CWD strain from sCJD, would serve as useful diagnostic tools in the characterization of

human prion diseases, especially those of novel phenotype. This would enable cases of human prion disease to be identified as sporadic, or derived from exposure to animal prions. Currently, no case of vCJD with a genotype other than methionine homozygous has been formally diagnosed. Because it is not known what the phenotype of vCJD in other genotypes may be, or what the phenotype of a CWD case might be, the ability to rapidly identify strain characteristics of PrP^{Sc} in this situation would be invaluable to surveillance efforts.

APPENDIX 1

Biography

Education

Bachelor of Science (Honors) 2003
Trent University, Peterborough, ON, Canada

Doctor of Philosophy 2009
Department of Pathology
Case Western Reserve University, Cleveland, OH

Abstracts and Publications

Hatcher K, Zheng J, Chen, SG. Cryptic Peptides of the Kringle Domains Preferentially Bind to Disease-Associated Prion Protein. Society for Neuroscience San Diego, CA. 2004

Hatcher K, Harris C, Gambetti P, Chen SG. (2005). Advances in Prion Disease Surveillance. Advances in Clinical Chemistry 41, 263.

Hatcher K, Zheng J, Chen SG. Cryptic Peptides of the Kringle Domains Preferentially Bind to Disease-Associated Prion Protein. Journal of Alzheimer's Disease. In press

Wang P*, Hatcher K*, Bartz J, Richt J, Liu H, Chen SG, Sreevatsan S. Aptamers selected against PrPSc show high specificity to prions from different species. In preparation.

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Awards and Honors

Dean's Honor Roll Trent University 1999-2003
President's Honor Roll Trent University 2003

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