N-TERMINUS (Y145STOP) FRAGMENT OF THE PRION PROTEIN PLAYS A ROLE IN PRION MISFOLDING

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Ahmed Mohamed Abd-Allah

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Dr. Srinand Sreevatsan, Adviser

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DEDICATION

This is dedicated to the martyrs of January 25th Egyptian revolution—the bravest people

ABSTRACT

Prion diseases are transmissible protein misfolding disorders in which misfolding of a host-encoded prion protein (PrP) occurs. They comprise of a group of distinct diseases in animals and humans, which show similar clinical and neuropathological changes. Human prion diseases can arise sporadically, be hereditary or be acquired. Sporadic human prion diseases include Cruetzfeldt-Jacob disease (CJD) and fatal insomnia. Genetic or familial prion diseases are caused by autosomal dominantly inherited mutations in the gene encoding for PrP^{C} and include familial or genetic CJD, fatal familial insomnia and Gerstmann-Sträussler-Scheinker syndrome. Acquired human prion diseases account for only 5% of cases of human prion disease. They include Kuru, iatrogenic CJD and a new variant form of CJD that is transmitted to humans from affected cattle via meat consumption. Despite considerable effort in understanding the structure of the prion protein, the precise role of different prion protein domains that may be important in basic misfolding is poorly understood. The linkage between *Prnp* mutations and hereditary prion disease provide support for the central role of PrP in pathogenesis, because the genetic disease can be propagated in an infectious way. One of the most intriguing disease-related mutations is the tyrosine to stop codon substitution at position 145; Y145Stop variant of prion protein. Y145Stop variant; PrP23-144, is the only truncated prion molecule that is linked to an autosomal dominant inherited genetic TSE, Gerstmann-Sträussler-Scheinker syndrome. This disease related mutation indicates an essential role of the N-terminus fragment of the prion protein in seeding and misfolding of the mammalian prions. Therefore, it was hypothesized that the N-terminus fragment of the prion protein plays a central role in the mechanism of prion conversion.

Studies were designed to test the hypothesis using a Protein Misfolding Cyclic Amplification (PMCA) assay in the presence or absence of preexisting prions. It was identified that recombinant Y145Stop had the propensity for spontaneous conversion to protease resistant isoforms. Systematic molecular investigations established that Y145Stop was able to induce PrP^{Sc} formation in cell culture in comparison to other recombinant PrP fragments and prions extracted from infected brain tissues. Misfolded Y145Stop showed similar kinetics as naturally occurring PrP^{Sc} in cell culture infectivity. Lastly, it was demonstrated that the toxicity of Y145Stop in the cell culture was correlated with apoptotic cell death. The toxic activity of the peptide was dependent on the activation of caspase and p38 MAP kinase pathway. These experiments established a critical role for Y145Stop molecule in prion conversion of recombinant and mammalian prions. Experimental evidence findings lead to propose a two-step phenomenon in prion misfolding whereby, the N-terminus first interacts with metals or polyanions leading to its misfolding that then catalyzes the conformational conversion of the structured C-terminus of the molecule. Taken together, data generated from these studies will provide better understanding of the prion conversion mechanism. Elucidating the role of the N-terminus in seeding and misfolding of mammalian prions has important implications for designing diagnostics and therapeutics of prion diseases, as well as for understanding pathogenic mechanisms operative in interspecies transmission.

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GENERAL INTRODUCTION

The central event in prion pathogenesis is the conformational conversion of PrP^{C} into PrP^{Sc}, an insoluble and partially protease-resistant isoform that self-propagates by imposing its abnormal conformation onto PrP^{C} molecules. Several in vitro conversion assays have been developed over the past two decades to investigate how PrP^{C} is conformationally altered by PrP^{Sc}. However, molecular conversion in various cell-free systems failed to completely reproduce the proposed prion conversion process. A possible reason for this may relate to the fact that the precise role of different molecular domains that may be important in basic misfolding is poorly understood. Since no other proteins are known to be involved in this conversion, the existence of a specific and probably efficient self-interaction between PrP molecules must be considered.

The linkage between *Prnp* mutations and hereditary prion disease provide support for the central role of PrP in pathogenesis. One of the most intriguing disease-related mutations is the tyrosine to stop codon substitution at position 145; Y145Stop variant of prion protein. This disease related mutation indicates an essential role of the N-terminus fragment of the prion protein in seeding and misfolding of the mammalian prions. Therefore, understanding how the N-terminus fragment of the prion protein affects biophysical properties of prion protein could provide insight into the molecular basis of the disease in general and the mechanism of prion protein conversion in particular. Therefore, it was hypothesized that the N-terminus fragment of the prion protein plays a central role in the mechanism of prion conversion.

The overall goal of these studies was to elucidate the role of N-terminus fragment of prion protein in generation of infectivity of prions. Therefore, experimental studies with three specific aims were designed.

Specific aim 1: Characterize the propensity of the different prion protein fragments for conformational conversion to amyloid fibrils *in vitro* conditions.

Specific aim 2: Determine the proficiency of the N-terminal of prion protein (Y145Stop) in generation of infectivity using a cell culture system.

Specific aim 3: Investigate the molecular aspects of the cell death machinery triggered by Y145Stop using a cell culture model.

This thesis is divided into four chapters: Chapter 1 provides a literature review with a focus on structural biology and mechanism of conversion of prion protein; Chapter 2 addresses the role of Y145Stop in catalyzing the conversion PrP^{C} into PrP^{Sc} using Protein Misfolding Cyclic Amplification (PMCA) assay. Chapter 3 examines infectivity of Y145Stop in an ex-vivo cellular model and its kinetics in comparison to infectious prions; Chapter 4 characterizes the intracellular signaling responsible for Y145Stop-dependent cell death in cell culture model.

CHAPTER 1: Literature Review

1. Overview of prion diseases.

Prion diseases comprise of a group of distinct diseases in animals and humans, which show similar clinical and neuropathological changes. Human prion diseases are unique in that they occur as either inherited or acquired forms. Sporadic Creutzfeldt-Jakob-disease (sCJD) is the most common form of prion disease that accounts for about 85% of spongiform encephalopathies in humans [1]. Approximately 15% of these are inherited and associated with coding mutations in the *Prnp* gene; include familial CJD (fCJD), familial fatal insomnia, and Gerstmann-Sträussler-Scheinker syndrome. The acquired forms include Kuru that has been shown to be transmitted via ritualistic cannibalism among the fore tribes of Papua New Guinea, iatrogenic CJD transmitted via contaminated corneal transplants or growth hormone administration, and variant CJD (vCJD) acquired by ingestion of bovine spongiform encephalopathy (BSE)-contaminated food. In animals, the main prion diseases include scrapie in sheep, BSE in cattle, and chronic wasting disease (CWD) in deer and elk. For decades, research has led to incremental advances toward understanding their disease mechanisms. The agent responsible for this group of diseases was first identified to be resistant to most destructive procedures that normally destroy nucleic acids, such as UV and ionizing radiation [2]. Therefore, the disease-causing agent, which also had been called a 'slow virus' [3] has now been identified as a mammalian protein designated, 'prion'.

The unique feature of prion diseases is that prions are self-propagating and transmissible [4]. Prion diseases are caused by misfolding of the normal prion protein designated as, PrP^{C}, a 35kD membrane glycoprotein that is water-soluble and proteinase-sensitive [5]. A conformational conversion to misfolded prions, designated as PrP^{Sc},

results in resistance proteases and causes its precipitation as insoluble amyloids leading to neuronal degeneration [6]. The mechanism of this conformational conversion remains poorly understood. Therefore, this study set out to study the role of the different regions of the molecule involved in misfolding and/or catalysis of the misfolding process. A better understanding of molecular interactions involved in prion misfolding will aid in the development of targeted therapeutics that may help improve quality of life of affected patients.

2. Research Milestones in Prion Disease

2.1. Prion Transmissibility

The first indication that prions were transmissible was reported in 1937 in Scotland after immunization of sheep against louping ill with a formalin extract of brain tissue derived from an animal with scrapie [7]. In the same year, Cullie and Chele demonstrated the experimental transmission of the agent among sheep and goats [8]. In humans, the successful transmission of Kuru, a neurodegenerative disease that affected the cannibalistic tribes of Papua New Guinea, to chimpanzees demonstrated similar infectious route to scrapie [9]. This was followed by transmission of CJD [10], and a familial form of TSE, Gerstmann-Sträussler-Scheinker (GSS) [11] to animals. Thus, both natural and experimental evidence suggest that prion diseases are infectious in nature and are capable of interspecies transmission.

2.2. Emergence of a protein-only hypothesis

Prior to the discovery of prions, it was thought that all pathogens require nucleic acids to direct their replication. Initially, the agent was thought to be slow virus, because of the long incubation period of the disease [3]. However, Alper and colleagues reported that a large amount of ionizing radiation was required for inactivation of the scrapie agent in tissue preparations [12]. Thus, the molecular weight of target was significantly lower by the order of 10^5 daltons, presumably the size of the scrapie agent [12]. Moreover, Alper showed that the scrapie agent was highly resistant to treatments that normally destroy the nucleic acids, such as UV and ionizing radiation [2]. These experiments led to the proposal by J. S. Griffith, a mathematician, that misfolded proteins could act as the infectious agent [13]. In the late eighties Weissmann's work showed that the absence of the PrP gene from the mouse prevented the disease and it was one of the first instances where knockout of mammalian gene had no readily discernible consequences and it earned the mouse protection against the disease [5]. This formulated the 'protein-only' hypothesis which was then extensively tested by Stanley Prusiner, who experimentally demonstrated that the prion was a small proteinaceous infectious particle [14].

2.3. The nature of the new infectious agent

One intriguing aspect of the prion diseases was the isolation of the disease-associated, protease-resistant protein (PrP^{res}) from the infectious material [15]. PrP^{res} infectivity was titratable in a mouse model [16]. Furthermore, the infectivity was reduced by agents that destroy protein structure, such as detergents and anti-PrP antibodies [16-18]. A crucial step in understanding the nature of the infectious agent was the discovery

and cloning of the *Prnp* gene that encoded PrP^{C} and proved to be a normal, cellular gene, present in the brain of animals [19-21]. Subsequent reports have shown that the PrP^{C} can be expressed in relative abundance in extraneuronal tissues, such as lymphocytes [22], follicular dendritic cell [23], platelets [24], and skeletal muscle [25]. Infectivity, although present in peripheral tissues, is at lower levels than in the central nervous system (CNS) [26]. This discovery guided the formulation of the prion hypothesis that postulates that the agent responsible for prion propagation originated by the autocatalytic conversion of normal cellular protein (termed PrP^{C}) into the pathogenic isoform (termed PrP^{res} or PrP^{Sc}). This conversion involves a conformational change of α-helices into β-sheets [27] followed by changes in other physicochemical properties, such as hydrophobicity and partial to complete protease resistance.

2.4. Genetic evidence supporting the prion hypothesis

Numerous experiments have provided evidence that PrP^{C} is a key player in prion replication as well as in prion-induced neurodegeneration. The linkage between *Prnp* mutations and hereditary prion disease provide support for the central role of PrP in pathogenesis, because the genetic disease can be propagated in an infectious way [28]. While mice lacking the prion gene are resistant to the disease [5], a TSE-like disease was produced in mice overexpressing PrP genes with point mutations linked to GSS syndrome [29]. These findings confirm that expression of PrP^{C} is necessary to initiate the disease.

2.5. Cell-free conversion of PrP^{C} into PrP^{res}

A significant milestone in support of the prion hypothesis was the establishment of an in-vitro conversion system to generate PrP^{res} from PrP^{C} in a cell-free environment. De-novo generation or amplification of misfolded prions from PrP^{C} proved the protein-only hypothesis. The original system was established by coincubation of substantially purified and radiolabeled PrP^{C} with stoichiometric amounts of purified PrP^{res} [30]. This work showed formation of radiolabeled PrP^{res} indicating the transformation of the normal protein into PrP^{res}. Nonetheless, this system required large amounts of PrP^{res} and produced a low yield of PrP^{res}, which preclude the detection of de novo prion formation. Soto and colleagues established protein misfolding cyclic amplification (PMCA) that utilizes cyclic bursts of sonication to convert PrP^{C} into a protease-resistant, infectious PrP^{Sc}-like product by incubation of a mixture of prion-infected brain homogenate diluted in a >1000 fold excess of normal, uninfected brain homogenate [31]. A major change in PMCA was achieved by the incorporation of a programmable sonicator and a 96-well plate format, which enabled high through-put assays [32]. Therefore, the invention of PMCA is considered as a major breakthrough in science and technology, because it allows to mimic in vitro the pathological process associated to these diseases in a rapid and efficient way and cemented the fact that infectious prions can be generated in a cell free system [31, 33].

3. Prion Biology

3.1. The structural biology of the cellular prion

The structure of PrP^C is highly conserved among species and throughout evolution, suggesting an important biological role [34]. In humans, PrP^C is approximately 250 amino acids with a 35 kDa molecular weight. During its biosynthesis, the molecule is subject to several kinds of posttranslational modification, including cleavage of an amino-terminal signal peptide, addition of N-linked oligosaccharide chains at asparagines 182 and 198, and formation of a single disulphide bond between cysteines 179 and 214. The asparagine glycosylation determines variations in the biochemistry of the mature protein resulting in di-, mono-, or un-glycosylated forms [35]. PrP^C is found as a mixture of these forms with variable proportions depending on the tissue and species of animal in question [35]. TSE strains display differences in abundance and electrophoretic mobility of the proteinase K (PK) resistant forms of PrP^{Sc} glycoforms and these differences have been used extensively as a method to discriminate between the various TSE strains, through a process known as glycotyping [6, 36, 37].

In addition, the prion molecule has a hydrophobic carboxy-terminal domain for membrane anchoring via a glycosylphosphatidylinositol (GPI) anchor. Like other GPI-linked proteins, it is enriched in detergent-resistant membranes. Although, the N-terminal of the molecule is not structured, it contains two defined and conserved domains. The first consists of five octapeptide repeat motifs, codons 60–91; (numbering based on human sequence, [38]) has been proposed to be important in copper binding and may be involved in pathogenesis [39, 40]. The other region, codon 113–135, contains a highly hydrophobic and conserved sequence. It has been suggested that this forms the

hydrophobic stretch sustaining PrP^{Sc} aggregate formation and may also sustain protein-membrane interactions during the reconstitution process [41-43]. Thus, it may exist as a transmembranous region in some PrP isoforms. Interestingly, there is a high conservation of sequences (residues 23–90) which are not associated with either the structural elements or the core infectious portion of the molecule. Fourier transformed infrared spectroscopy (FTIR) and circular dichroism (CD) indicated that PrP^{C} had a high α-helical content (~42%) and a relatively small β-sheet content (~3%) [27, 44, 45]. In contrast to the NH_2-proximal half of the molecule with no secondary structure, the COOH-proximal half is arranged in three α-helices corresponding, for the human PrP^{C}, to the residues 144–154, 173–194, and 200–228, interspersed with two β-sheets formed at residues 128–131 and 161–164 [38, 45, 46]. Because the transition of PrP^{C} to PrP^{Sc} involves a major increase in the β-sheet content (~40%) and reduction in the α-helix content (~30%) [27, 47, 48], the data indicate that this conformational conversion involves regions of the random coils to β-sheet. Therefore, the amino-terminal region affects the global secondary structure and behaviour of the carboxy-terminal segment.

3.2. Physiological Function of PrP^{C}

Despite advances in the characterization of PrP^{C} structure, the function of PrP^{C} remains enigmatic. Many different functions of PrP^{C} have been proposed, including signal transduction, copper binding, synaptic transmission and many others [49]. Some studies have suggested a cytoprotective of PrP^{C} against oxidative stress and apoptosis stimuli. Neurons isolated from *Prnp* knockout mice showed higher susceptibility to oxidative agents compared to wild-type neurons [50, 51]. It has been shown that PrP^{C} can

bind copper via the octapeptide repeat region [52, 53]. The binding of copper to PrP^C may limit the formation of reactive oxygen species catalyzed by free copper [54, 55], or modulate the activity of the Cu/Zn superoxide dismutase enzyme that show cellular protective function against oxidative stress [50, 56]. Although, it has been suggested that the N-terminal truncated PrP^C may suppress endogenous dismutase activity [57], PrP^C did not provide any measurable contribution to dismutase activity in vivo [58]. A cytoprotective role of PrP^C against internal or environmental stresses that initiate apoptosis has been proposed. This anti-apoptotic potential is based on the capacity of PrP^C to inhibit the action of pro-apoptotic protein Bax [59] that affects the release of cytochrome c. Other studies have reported an analogy in the protein structure between the anti-apoptotic protein Bcl-2 and the PrP^C octapeptide region. Here the protein structure may allow PrP^C to mimic Bcl-2 function and induce cell survival [60, 61]. Importantly, PrP^C dimerization by delivery of monoclonal PrP antibodies in vivo induces an apoptotic signal [62]. However, the molecular mechanisms underlying these findings have not been elucidated. Studies using *Prnp* knockout mice have failed to explicate the molecular function of the protein. The only well-defined phenotype of the knockout mice was their resistance to the prion disease [5] with subtle abnormalities [63-65]. Therefore, it is unlikely that prion pathology occurs due to simple loss of PrP^C function, because *Prnp* ablation did not elicit disease [66]. In recent studies, a zebrafish model has been used to show that PrP provides cellular signals that regulate cell communication in vivo. PrP knockdown in zebrafish results in a loss of embryonic cell adhesion and arrested gastrulation [67]. In this study, it was demonstrated that PrP modulates Ca^{+2}-dependent cell adhesion by regulating the delivery of E-cadherin to the plasma membrane.

Interestingly, both zebrafish and mouse PRNP mRNAs could rescue the knockdown phenotype, indicating an evolutionarily conserved prion protein function. Investigating the normal function of PrP^{C} is critical for understanding the pathogenesis of prion disease, since alteration of this function could play a role in the disease process. It is important to identify the physiological relevant PrP-interacting partners, and the cellular pathways in which they participate. Whatever the function of PrP^{C}, the conversion to PrP^{Sc} leads to its alteration and this begins a cascade of yet unclear molecular events that result in neuropathology [68].

3.3. Mechanism of Prion conversion

During the last few years, research into prion biology has mainly focused on determination of the pathogenesis of TSEs and the development of diagnostic and therapeutic methods. The central event in prion pathogenesis is the conformational conversion of PrP^{C} into PrP^{Sc}, an insoluble and partially protease-resistant isoform that self-propagates by imposing its abnormal conformation onto PrP^{C} molecules. Two distinct models have been proposed to elucidate the autocatalytic conversion of PrP^{C}. The heterodimer or template directed refolding model [69] supposes that PrP^{C} monomers would be unfolded to some extent and subsequently refolded under the influence of the incoming PrP^{Sc} which would behave as a chaperone. In this model, the conversion is mediated by an intermediate (PrP^{*}) and heterodimeric unit before the formation of the stable homodimeric PrP^{Sc}. High activation energy is the barrier that controls the conformational change, but formation of the heterodimeric complex may lower that barrier. The alternative model, known as the noncatalytic nucleation model or (seeded

polymerization model) [30, 70, 71] proposes that the conversion of PrP^{C} and PrP^{Sc} as a reversible process, but strongly favors the conformation of PrP^{C} when at equilibrium. Polymerization of several PrP^{Sc} monomers is required to form a nucleus and stabilize the converted PrP^{Sc} a crystal-like seed form. Once the nucleus (of a critical size) has been formed, faster additions occur through the binding of PrP^{C} into the polymer. Furthermore, recent studies indicate that the most infectious particle per unit PrP has an average mass equivalent to a 14−28-mer with the minimal infectious unit being larger than a 5-mer [72]. Whether prions multiply by template-directed refolding or by the nucleation model, certain domains of PrP^{C} would be required to interact with oligomeric seeds and rearrange such that the monomeric protein becomes capable of inducing the same change in further PrP^{C} monomers. In addition to the models in support of the prion hypothesis, alternative models suggesting the participation of viral particles, virinos and small RNAs have been also proposed. The virino model describes that the causative agent of TSE is a proteinaceous structure containing nucleic acids with a virus-like conformation [73]. The fact that prions have different strains has been used as evidence to support this model [73]. However, no evidence for TSE-specific nucleic acids has yet been identified [49, 74, 75] and the strain phenomenon can be explained by the variation in PrP^{Sc} protein secondary structure [4]. Another theory, the bacterial theory, proposes that a tiny, wall-less bacteria called spiroplasma attaches to normal prion protein in the brain and causes it to misfold [76]. However, the failure of a blinded study of rRNA species to detect any footprint of Spiroplasma in scrapie-infected hamster brain laid it to rest [77].

Over the past years, accumulated evidence has supported the protein-only hypothesis. Final proof of hypothesis is to show that pure PrP^{C} or recombinant PrP can be

converted into a form that recapitulates the pathology of prion disease. In 2004, Legname et al. [78] reported the production of "synthetic prions" via conversion of bacterially expressed recombinant prion protein (rPrP) into amyloid fibrils. Intracerebral inoculation of these amyloid fibrils into transgenic mice overexpressing PrP induced a transmissible neurodegenerative disease with a very long incubation period. Further studies have shown that TSE infectivity was accomplished by protein misfolding cyclic amplification (PMCA), a technique by which PrP^{Sc} is amplified by cycles of sonication followed by incubation with brain homogenate [31, 79]. Soto and coworkers showed that amplified PrP^{Sc} was infectious to wild-type hamster [33]. However, the use of purified PrP^{C} instead of brain homogenate as a substrate decreased the conversion efficacy [80, 81]. Recently, amplification of TSE infectivity was also accomplished by PMCA by employing purified PrP^{C} as a substrate [82, 83], exclusively in presence of polyanions. These data suggest that additional cofactors may be required to facilitate the conversion of the recombinant forms of PrP.

Given the interest in the infectious aspects of prions, the elucidation of the cellular mechanisms involved in spontaneous PrP misfolding and formation of an intermediate complex is a critical aspect. Recent studies have shown that certain aspects of the prion conversion mechanism can be studied by using the recombinant Y145Stop mutant of the human protein, huPrP23-144 [39, 84, 85]. This pathogenic truncation mutation (amber) is the only known truncated protein that is linked to a prion disease, Gerstmann-Sträussler-Scheinker (GSS) [86, 87]. Since GSS is an autosomal dominant disorder, mutant PrP^{c} molecules might spontaneously convert into mutant PrP^{Sc}. Thus, one mutant allele with the Y145Stop is able to recruit and seed the 'other' allele [88]. It was found that

recombinant fragment residues (23-144) can undergo very efficient autocatalytic conformational conversion under physiologically relevant buffer conditions [39, 84]. However, a similar reaction for recombinant full-length prion protein or PrP90-231 has been reported only in presence of high concentrations of chemical denaturants [89, 90]. Most importantly, it has been shown that phenomena of "species barrier" and prion strains can be reproduced using the Y145Stop model [91, 92]. Taken together these data suggest an essential role of the N-terminus of the molecule, spanning amino acids 23-144, in the mechanism of prion misfolding as well as in protein-protein interactions. PrP^{C} appears to bind to PrP^{Sc}, possibly in combination with auxiliary proteins, to form an intermediate complex during the formation of de novo PrP^{Sc} [93]. In the absence of preexisting PrP^{Sc}, several studies have shown that a variety of molecules, such as RNA [94], DNA [95], and heavy metals including copper [96, 97] appear to stimulate prion conversion. The N-terminal fragment of PrP has been shown to bind with copper [98], DNA [99, 100], and RNA [101] whereas the C-terminal fragment (122-231) does not demonstrate a similar binding affinity to these molecules. Based on these observations, we propose a two-step phenomenon in prion misfolding. In the first instance, any one of these cofactors maybe involved in binding to and misfolding of the N-terminal region of the molecule, which then likely catalyzes conversion of the full prion molecule. Therefore, the misfolded N-terminal region could be the intermediate (PrP^{*}) in the heterodimer model or in the unstable monomer of the nucleation model, which mediates the formation of nascent PrP^{Sc}.

4. Prion Diversity (prion strains & species barriers)

Prions strains are defined as infectious isolates that impose, after inoculation in an autologous host, specific and stable phenotypes such as incubation period, molecular pattern of PrP^{Sc} and neuropathology. The prion diversity was initially demonstrated in goats with "hyper" and "drowsy" isolates [102]. Subsequent studies in rodents have demonstrated the existence of at least 14 scrapie strains with characteristic phenotypes [103-107]. Interestingly, new distinct strains can be observed upon transmission of prions across an interspecies barrier [105, 108]. Cross-species transmission studies, typically to laboratory rodents, have showed that species barrier is abrogated after a few subpassages, reflecting adaptation to its new host [109]. The recent evidence of BSE in humans, termed as vCJD has highlighted the ability of prions to cross and propagate in other species, which might constitute a risk for human health. Furthermore, Nor98, 'atypical' form of scrapie has been discovered more recently in sheep and goat through active surveillance [110]. In this form, the abnormal PrP is characterized by a lower resistance to protease digestion as compared to classical scrapie [110]. In the absence of TSE-specific nucleic acids [49, 74, 75], the prion strains diversity could be explained by the existence of variable PrP^{Sc} conformations. Although it is well known that conformational variation of PrP-res present in both in vivo and in vitro situations, little is understood about the molecular contributions of different regions of PrP to this variation. Indeed prion strains differ in their biochemical properties, which include electrophoretic mobility after proteinase K digestion that reflects a different access to the N-terminal region of the PrP molecule, the ratio of glycoforms and the stability toward denaturing agents. However, the molecular basis and the mechanism by which differences in PrP^{Sc}

glycosylation states affect the disease remain unclear. In cell-free assays, PrP^{C} conversion product retains a PrP^{res} molecular signature similar to that of seeding material [111, 112]. In some CJD cases, the coexistence of multiple PrP^{Sc} types has been shown [113]. Furthermore, experimental studies have shown that when two strains infect the same host, one strain can suppress the ability of the second strain to cause disease [114, 115].

Early studies suggested that the host and strain properties are the major determinants for species barriers [116, 117]. Therefore, the degree of susceptibility for the prion strains varies among different species. The major determinants for a crossspecies barrier resides essentially in PrP primary structural differences between the host and donor species. Scott et al. [118] abrogated the known resistance of the mouse to hamster scrapie [119] by expressing hamster PrPC in transgenic mice. Albeit, the PrP sequence as well as the tridimensional PrP^{C} structure [120] is highly conserved among mammalian species, the minimal amino acid divergences may have a major effect on the transmission efficiency. It is known that a polymorphism at residue 129 (M or V) of human PrP influences susceptibility to human TSE, with methionine homozygosity a predisposing factor to the development of CJD. Moreover, sheep breeds exhibit variable susceptibilities to experimental or natural scrapie based on polymorphisms at codons 136, 154 and 171 of the ovine PrP gene [121, 122]. Sheep homozygous for the $V_{136}R_{154}Q_{171}$ (VRQ) allele are highly susceptible to classical scrapie whereas sheep homozygous for $A_{136}R_{154}R_{171}$ (ARR) exhibit marked resistance [123, 124]. Atypical scrapie has been diagnosed in sheep carrying various PrP genotypes, including those homozygous for ARR [125-127].

Therefore, at the molecular level, much remains to be learned about the physical relationship between infectivity and misfolded PrP particles, and to which extent it varies according to the PrP sequence and/or prion strain [128]. We hypothesize that minor variations in the structural portion of the prion molecule, PrP 127-227, may reflect the ease of conversion suggesting the proposed two-step phenomenon in prion misfolding.

5. Mechanisms of Prion Toxicity

The requirement of PrP^{C} expression for infection and toxicity, suggests prion disease may be due to, at least in part, interference with the function of PrP^{C}. However, the study of PrP knockout mice, argue against the simple loss of PrP^{C} function [129]. There is a possibility that PrP null mice do not develop neurodegeneration due to compensatory adaptations [66]. Alternatively, the toxicity of PrP^{Sc} might depend on some PrP^{C}-dependent process leads to neuronal death [130, 131]. A number of studies indicate that neuronal dysfunction and death in humans and animals affected with prions occurs through apoptosis [132-140]. It has been shown that neurons derived from *Prnp*-deficient mice were more susceptible to apoptosis. This effect could be rescued by the expression of either B-cell lymphoma protein 2 (Bcl-2) or PrP^{C} [141]. The BH2 domain of the BCL-2 proteins has an anti-apoptotic role through its interaction with the pro-apoptotic Bax protein [142, 143]. Kurschner and Morgan reported that yeast PrP fusion proteins interact with BCL-2. Furthermore, four N-terminal PrP octapeptide repeats (OR) that are highly conserved share limited similarity with the BH2 of Bcl-2 proteins [144, 145]. These findings suggested that PrP may play a role in the regulation of the neuronal apoptosis.

This interpretation finds support from Solforosi et al., who showed that cross-linking of PrP^{C} by a monoclonal antibody results in rapid and extensive apoptosis [62].

PrP^{Sc} is derived from PrP^{C} by a post-translational mechanism [146], suggesting that *in vitro* generation of prions from highly purified recombinant PrP should be possible. However, several studies suggest that cellular factors, other than those added *in vitro*, are necessary for the acquisition of the infectious properties [30, 147-149]. Due to the difficulty in isolating pure infectious PrP^{Sc}, synthetic PrP peptides are utilized to model prion disease *in vivo* and unravel the mysteries associated with PrP^{Sc} infectivity and toxicity. Forloni et al. created a synthetic peptide homologous to a highly conserved region of the prion protein, residues 106-126 (PrP106-126), that exhibited physiochemical properties resembling PrP^{Sc} [150]. The synthetic peptide was able to form amyloid and trigger neuronal death by apoptosis [150]. On the other hand, transgenic mice expressing PrP fragments die spontaneously by ataxia, showing accumulation of protease resistance PrP (PrP^{res}), apparently causing apoptosis [151, 152]. Moreover, deletions of amino acids 32–121 or 32–134 (termed ΔPrP) confer strong neurotoxicity to PrP^{C} in vivo, which indicates that ΔPrP is a functional antagonist of PrP^{C} [153, 154]. Further analysis of mice expressing PrP variants may allow for identification of functionally relevant domains within PrP^{C}. Alternatively, abnormal topology or altered trafficking of PrP^{C} has also been proposed to explain the PrP^{C}-related neuronal toxicity. PrP possesses a C-terminal GPI-anchoring signal and a transmembrane domain that can generate type I (^{Ctm}PrP) or type II (^{Ntm}PrP) transmembrane-spanning isoforms [155-158]. In the absence of TrAF, translocation accessory factors, PrP is synthesized in a transmembrane topology[158].

Thus the propensity for the formation of transmembrane intermediates might lead to neurotoxicity in the absence of PrP^{Sc} formation.

Concluding Remarks and Future Directions

Prion diseases are disorders of protein misfolding, and elucidating their precise molecular mechanisms will have implications beyond TSE disorders, but also for more common neurodegenerative diseases such as Alzheimer's and Parkinson's disease. It is of considerable interest that a single polypeptide chain can apparently encode information and specify distinctive phenotypes. Although the protein-only hypothesis of prion propagation is supported by compelling experimental data, many open questions in the prion field remain. It is important to understand prion conversion mechanisms and how strain information is maintained and transmitted. PrP^{Sc}-like forms of PrP have been produced from purified recombinant material, but the reason for the poor infectivity of these forms in experimental animals is not clear. Another aim is to elucidate the physiological function of PrP^{C} and how neurotoxicity is induced by the prion agent. The ability to answer these questions in the future will rely mainly on the quality and resolution of the tools and technologies available to the prion field.

REFERENCES

1. Hill, A.F., et al., *Molecular classification of sporadic Creutzfeldt-Jakob disease.* Brain, 2003. **126**(Pt 6): p. 1333-46.
2. Alper, T., et al., *Does the agent of scrapie replicate without nucleic acid?* Nature, 1967. **214**(5090): p. 764-6.
3. Cho, H.J., *Is the scrapie agent a virus?* Nature, 1976. **262**(5567): p. 411-2.
4. Prusiner, S.B., *Prions.* Proc Natl Acad Sci U S A, 1998. **95**(23): p. 13363-83.
5. Bueler, H., et al., *Mice devoid of PrP are resistant to scrapie.* Cell, 1993. **73**(7): p. 1339-47.
6. Casalone, C., et al., *Identification of a second bovine amyloidotic spongiform encephalopathy: molecular similarities with sporadic Creutzfeldt-Jakob disease.* Proc Natl Acad Sci U S A, 2004. **101**(9): p. 3065-70.
7. Gordon, W.S., *Advances in veterinary research.* Vet Rec, 1946. **58**(47): p. 516-25.
8. Cullie, J., and Chelle, P.L., *Experimental transmission of trembling to the goat.* C. R. Seances Acad. Sci., 1939. **208**: p. 1058-1160.
9. Gajdusek, D.C., C.J. Gibbs, and M. Alpers, *Experimental transmission of a Kuru-like syndrome to chimpanzees.* Nature, 1966. **209**(5025): p. 794-6.
10. Gibbs, C.J., Jr., et al., *Creutzfeldt-Jakob disease (spongiform encephalopathy): transmission to the chimpanzee.* Science, 1968. **161**(3839): p. 388-9.
11. Masters, C.L., D.C. Gajdusek, and C.J. Gibbs, Jr., *Creutzfeldt-Jakob disease virus isolations from the Gerstmann-Straussler syndrome with an analysis of the various forms of amyloid plaque deposition in the virus-induced spongiform encephalopathies.* Brain, 1981. **104**(3): p. 559-88.
12. Alper, T., D.A. Haig, and M.C. Clarke, *The exceptionally small size of the scrapie agent.* Biochem Biophys Res Commun, 1966. **22**(3): p. 278-84.
13. Griffith, J.S., *Self-replication and scrapie.* Nature, 1967. **215**(5105): p. 1043-4.
14. Prusiner, S.B., *Novel proteinaceous infectious particles cause scrapie.* Science, 1982. **216**(4542): p. 136-44.
15. Bolton, D.C., M.P. McKinley, and S.B. Prusiner, *Identification of a protein that purifies with the scrapie prion.* Science, 1982. **218**(4579): p. 1309-11.
16. Gabizon, R., et al., *Immunoaffinity purification and neutralization of scrapie prion infectivity.* Proc Natl Acad Sci U S A, 1988. **85**(18): p. 6617-21.
17. Enari, M., E. Flechsig, and C. Weissmann, *Scrapie prion protein accumulation by scrapie-infected neuroblastoma cells abrogated by exposure to a prion protein antibody.* Proc Natl Acad Sci U S A, 2001. **98**(16): p. 9295-9.
18. Peretz, D., et al., *Antibodies inhibit prion propagation and clear cell cultures of prion infectivity.* Nature, 2001. **412**(6848): p. 739-43.
19. Basler, K., et al., *Scrapie and cellular PrP isoforms are encoded by the same chromosomal gene.* Cell, 1986. **46**(3): p. 417-28.
20. Chesebro, B., et al., *Identification of scrapie prion protein-specific mRNA in scrapie-infected and uninfected brain.* Nature, 1985. **315**(6017): p. 331-3.
21. Oesch, B., et al., *A cellular gene encodes scrapie PrP 27-30 protein.* Cell, 1985. **40**(4): p. 735-46.

22. Durig, J., et al., *Differential constitutive and activation-dependent expression of prion protein in human peripheral blood leucocytes.* Br J Haematol, 2000. **108**(3): p. 488-95.
23. McCulloch, L., et al., *Follicular dendritic cell-specific prion protein (PrP) expression alone is sufficient to sustain prion infection in the spleen.* PLoS Pathog, 2011. **7**(12): p. e1002402.
24. Perini, F., et al., *PrP27-30 is a normal soluble prion protein fragment released by human platelets.* Biochem Biophys Res Commun, 1996. **223**(3): p. 572-7.
25. Bosque, P.J., et al., *Prions in skeletal muscle.* Proc Natl Acad Sci U S A, 2002. **99**(6): p. 3812-7.
26. Ramasamy, I., et al., *Organ distribution of prion proteins in variant Creutzfeldt-Jakob disease.* Lancet Infect Dis, 2003. **3**(4): p. 214-22.
27. Pan, K.M., et al., *Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins.* Proc Natl Acad Sci U S A, 1993. **90**(23): p. 10962-6.
28. Hsiao, K., et al., *Linkage of a prion protein missense variant to Gerstmann-Straussler syndrome.* Nature, 1989. **338**(6213): p. 342-5.
29. Hsiao, K.K., et al., *Spontaneous neurodegeneration in transgenic mice with mutant prion protein.* Science, 1990. **250**(4987): p. 1587-90.
30. Kocisko, D.A., et al., *Cell-free formation of protease-resistant prion protein.* Nature, 1994. **370**(6489): p. 471-4.
31. Saborio, G.P., B. Permanne, and C. Soto, *Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding.* Nature, 2001. **411**(6839): p. 810-3.
32. Castilla, J., et al., *Protein misfolding cyclic amplification for diagnosis and prion propagation studies.* Methods Enzymol, 2006. **412**: p. 3-21.
33. Castilla, J., et al., *In vitro generation of infectious scrapie prions.* Cell, 2005. **121**(2): p. 195-206.
34. Gossert, A.D., et al., *Prion protein NMR structures of elk and of mouse/elk hybrids.* Proc Natl Acad Sci U S A, 2005. **102**(3): p. 646-50.
35. Russelakis-Carneiro, M., et al., *Changes in the glycosylation pattern of prion protein in murine scrapie. Implications for the mechanism of neurodegeneration in prion diseases.* J Biol Chem, 2002. **277**(39): p. 36872-7.
36. Biacabe, A.G., et al., *Distinct molecular phenotypes in bovine prion diseases.* EMBO Rep, 2004. **5**(1): p. 110-5.
37. Collinge, J., et al., *Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD.* Nature, 1996. **383**(6602): p. 685-90.
38. Riek, R., et al., *NMR characterization of the full-length recombinant murine prion protein, mPrP(23-231).* FEBS Lett, 1997. **413**(2): p. 282-8.
39. Moore, R.A., et al., *Octapeptide repeat insertions increase the rate of protease-resistant prion protein formation.* Protein Sci, 2006. **15**(3): p. 609-19.
40. Brockes, J.P., *Topics in prion cell biology.* Curr Opin Neurobiol, 1999. **9**(5): p. 571-7.
41. Gabizon, R. and S.B. Prusiner, *Prion liposomes.* Biochem J, 1990. **266**(1): p. 1-14.
42. Prusiner, S.B., *Scrapie prions.* Annu Rev Microbiol, 1989. **43**: p. 345-74.

43. Gasset, M., et al., *Predicted alpha-helical regions of the prion protein when synthesized as peptides form amyloid.* Proc Natl Acad Sci U S A, 1992. **89**(22): p. 10940-4.
44. Hornemann, S., et al., *Recombinant full-length murine prion protein, mPrP(23-231): purification and spectroscopic characterization.* FEBS Lett, 1997. **413**(2): p. 277-81.
45. Riek, R., et al., *NMR structure of the mouse prion protein domain PrP(121-231).* Nature, 1996. **382**(6587): p. 180-2.
46. Zahn, R., et al., *NMR solution structure of the human prion protein.* Proc Natl Acad Sci U S A, 2000. **97**(1): p. 145-50.
47. Morillas, M., D.L. Vanik, and W.K. Surewicz, *On the mechanism of alpha-helix to beta-sheet transition in the recombinant prion protein.* Biochemistry, 2001. **40**(23): p. 6982-7.
48. Safar, J., et al., *Thermal stability and conformational transitions of scrapie amyloid (prion) protein correlate with infectivity.* Protein Sci, 1993. **2**(12): p. 2206-16.
49. Aguzzi, A. and M. Polymenidou, *Mammalian prion biology: one century of evolving concepts.* Cell, 2004. **116**(2): p. 313-27.
50. Brown, D.R., R.S. Nicholas, and L. Canevari, *Lack of prion protein expression results in a neuronal phenotype sensitive to stress.* J Neurosci Res, 2002. **67**(2): p. 211-24.
51. Wong, B.S., et al., *Increased levels of oxidative stress markers detected in the brains of mice devoid of prion protein.* J Neurochem, 2001. **76**(2): p. 565-72.
52. Brown, D.R., et al., *The cellular prion protein binds copper in vivo.* Nature, 1997. **390**(6661): p. 684-7.
53. Wadsworth, J.D., et al., *Strain-specific prion-protein conformation determined by metal ions.* Nat Cell Biol, 1999. **1**(1): p. 55-9.
54. Martins, V.R., et al., *Insights into the physiological function of cellular prion protein.* Braz J Med Biol Res, 2001. **34**(5): p. 585-95.
55. Vassallo, N. and J. Herms, *Cellular prion protein function in copper homeostasis and redox signalling at the synapse.* J Neurochem, 2003. **86**(3): p. 538-44.
56. Wechselberger, C., et al., *The physiological functions of prion protein.* Exp Cell Res, 2002. **281**(1): p. 1-8.
57. Sakudo, A., et al., *Impairment of superoxide dismutase activation by N-terminally truncated prion protein (PrP) in PrP-deficient neuronal cell line.* Biochem Biophys Res Commun, 2003. **308**(3): p. 660-7.
58. Hutter, G., F.L. Heppner, and A. Aguzzi, *No superoxide dismutase activity of cellular prion protein in vivo.* Biol Chem, 2003. **384**(9): p. 1279-85.
59. Bounhar, Y., et al., *Prion protein protects human neurons against Bax-mediated apoptosis.* J Biol Chem, 2001. **276**(42): p. 39145-9.
60. Roucou, X., et al., *Cellular prion protein inhibits proapoptotic Bax conformational change in human neurons and in breast carcinoma MCF-7 cells.* Cell Death Differ, 2005. **12**(7): p. 783-95.
61. Westergard, L., H.M. Christensen, and D.A. Harris, *The cellular prion protein (PrP(C)): its physiological function and role in disease.* Biochim Biophys Acta, 2007. **1772**(6): p. 629-44.

62. Solforosi, L., et al., *Cross-linking cellular prion protein triggers neuronal apoptosis in vivo.* Science, 2004. **303**(5663): p. 1514-6.
63. Collinge, J., et al., *Prion protein is necessary for normal synaptic function.* Nature, 1994. **370**(6487): p. 295-7.
64. Nishida, N., et al., *A mouse prion protein transgene rescues mice deficient for the prion protein gene from purkinje cell degeneration and demyelination.* Lab Invest, 1999. **79**(6): p. 689-97.
65. Tobler, I., et al., *Altered circadian activity rhythms and sleep in mice devoid of prion protein.* Nature, 1996. **380**(6575): p. 639-42.
66. Mallucci, G.R., et al., *Post-natal knockout of prion protein alters hippocampal CA1 properties, but does not result in neurodegeneration.* EMBO J, 2002. **21**(3): p. 202-10.
67. Malaga-Trillo, E., et al., *Regulation of embryonic cell adhesion by the prion protein.* PLoS Biol, 2009. **7**(3): p. e55.
68. Aguzzi, A. and M. Heikenwalder, *Prion diseases: Cannibals and garbage piles.* Nature, 2003. **423**(6936): p. 127-9.
69. Prusiner, S.B., *Molecular biology of prion diseases.* Science, 1991. **252**(5012): p. 1515-22.
70. Come, J.H., P.E. Fraser, and P.T. Lansbury, Jr., *A kinetic model for amyloid formation in the prion diseases: importance of seeding.* Proc Natl Acad Sci U S A, 1993. **90**(13): p. 5959-63.
71. Kocisko, D.A., et al., *Species specificity in the cell-free conversion of prion protein to protease-resistant forms: a model for the scrapie species barrier.* Proc Natl Acad Sci U S A, 1995. **92**(9): p. 3923-7.
72. Silveira, J.R., et al., *The most infectious prion protein particles.* Nature, 2005. **437**(7056): p. 257-61.
73. Chesebro, B., *BSE and prions: uncertainties about the agent.* Science, 1998. **279**(5347): p. 42-3.
74. Prusiner, S.B. and S.J. DeArmond, *Molecular biology and pathology of scrapie and the prion diseases of humans.* Brain Pathol, 1991. **1**(4): p. 297-310.
75. Safar, J.G., et al., *Search for a prion-specific nucleic acid.* J Virol, 2005. **79**(16): p. 10796-806.
76. Bastian, F.O., et al., *Spiroplasma spp. from transmissible spongiform encephalopathy brains or ticks induce spongiform encephalopathy in ruminants.* J Med Microbiol, 2007. **56**(Pt 9): p. 1235-42.
77. Alexeeva, I., et al., *Absence of Spiroplasma or other bacterial 16s rRNA genes in brain tissue of hamsters with scrapie.* J Clin Microbiol, 2006. **44**(1): p. 91-7.
78. Legname, G., et al., *Synthetic mammalian prions.* Science, 2004. **305**(5684): p. 673-6.
79. Saa, P., J. Castilla, and C. Soto, *Ultra-efficient replication of infectious prions by automated protein misfolding cyclic amplification.* J Biol Chem, 2006. **281**(46): p. 35245-52.
80. Deleault, N.R., et al., *Protease-resistant prion protein amplification reconstituted with partially purified substrates and synthetic polyanions.* J Biol Chem, 2005. **280**(29): p. 26873-9.

81. Atarashi, R., et al., *Ultrasensitive detection of scrapie prion protein using seeded conversion of recombinant prion protein.* Nat Methods, 2007. **4**(8): p. 645-50.
82. Wang, F., et al., *Generating a prion with bacterially expressed recombinant prion protein.* Science, 2010. **327**(5969): p. 1132-5.
83. Deleault, N.R., et al., *Formation of native prions from minimal components in vitro.* Proc Natl Acad Sci U S A, 2007. **104**(23): p. 9741-6.
84. Kundu, B., et al., *Nucleation-dependent conformational conversion of the Y145Stop variant of human prion protein: structural clues for prion propagation.* Proc Natl Acad Sci U S A, 2003. **100**(21): p. 12069-74.
85. Abdallah, A., et al., *Y145Stop is sufficient to induce de novo generation prions using protein misfolding cyclic amplification.* Prion, 2012. **6**(1).
86. Kitamoto, T., R. Iizuka, and J. Tateishi, *An amber mutation of prion protein in Gerstmann-Straussler syndrome with mutant PrP plaques.* Biochem Biophys Res Commun, 1993. **192**(2): p. 525-31.
87. Ghetti, B., et al., *Vascular variant of prion protein cerebral amyloidosis with tau-positive neurofibrillary tangles: the phenotype of the stop codon 145 mutation in PRNP.* Proc Natl Acad Sci U S A, 1996. **93**(2): p. 744-8.
88. Kitamoto, T. and J. Tateishi, *Human prion disease and human prion protein disease.* Curr Top Microbiol Immunol, 1996. **207**: p. 27-34.
89. Bocharova, O.V., et al., *In vitro conversion of full-length mammalian prion protein produces amyloid form with physical properties of PrP(Sc).* J Mol Biol, 2005. **346**(2): p. 645-59.
90. Apetri, A.C., D.L. Vanik, and W.K. Surewicz, *Polymorphism at residue 129 modulates the conformational conversion of the D178N variant of human prion protein 90-231.* Biochemistry, 2005. **44**(48): p. 15880-8.
91. Vanik, D.L., K.A. Surewicz, and W.K. Surewicz, *Molecular basis of barriers for interspecies transmissibility of mammalian prions.* Mol Cell, 2004. **14**(1): p. 139-45.
92. Jones, E.M. and W.K. Surewicz, *Fibril conformation as the basis of species- and strain-dependent seeding specificity of mammalian prion amyloids.* Cell, 2005. **121**(1): p. 63-72.
93. Meier, P., et al., *Soluble dimeric prion protein binds PrP(Sc) in vivo and antagonizes prion disease.* Cell, 2003. **113**(1): p. 49-60.
94. Deleault, N.R., R.W. Lucassen, and S. Supattapone, *RNA molecules stimulate prion protein conversion.* Nature, 2003. **425**(6959): p. 717-20.
95. Cordeiro, Y., et al., *DNA converts cellular prion protein into the beta-sheet conformation and inhibits prion peptide aggregation.* J Biol Chem, 2001. **276**(52): p. 49400-9.
96. Quaglio, E., R. Chiesa, and D.A. Harris, *Copper converts the cellular prion protein into a protease-resistant species that is distinct from the scrapie isoform.* J Biol Chem, 2001. **276**(14): p. 11432-8.
97. Leclerc, E., et al., *Copper induces conformational changes in the N-terminal part of cell-surface PrPC.* Arch Virol, 2006. **151**(11): p. 2103-9.
98. Viles, J.H., et al., *Copper binding to the prion protein: structural implications of four identical cooperative binding sites.* Proc Natl Acad Sci U S A, 1999. **96**(5): p. 2042-7.

99. Nandi, P.K., *Interaction of prion peptide HuPrP106-126 with nucleic acid.* Arch Virol, 1997. **142**(12): p. 2537-45.
100. Takemura, K., et al., *DNA aptamers that bind to PrP(C) and not PrP(Sc) show sequence and structure specificity.* Exp Biol Med (Maywood), 2006. **231**(2): p. 204-14.
101. Gabus, C., et al., *The prion protein has RNA binding and chaperoning properties characteristic of nucleocapsid protein NCP7 of HIV-1.* J Biol Chem, 2001. **276**(22): p. 19301-9.
102. Pattison, I.H. and G.C. Millson, *Scrapie produced experimentally in goats with special reference to the clinical syndrome.* J Comp Pathol, 1961. **71**: p. 101-9.
103. Bruce, M.E. and A.G. Dickinson, *Biological evidence that scrapie agent has an independent genome.* J Gen Virol, 1987. **68 (Pt 1)**: p. 79-89.
104. Dickinson, A.G. and G.W. Outram, *Genetic aspects of unconventional virus infections: the basis of the virino hypothesis.* Ciba Found Symp, 1988. **135**: p. 63-83.
105. Bruce, M.E., *Scrapie strain variation and mutation.* Br Med Bull, 1993. **49**(4): p. 822-38.
106. Bruce, M.E., *TSE strain variation.* Br Med Bull, 2003. **66**: p. 99-108.
107. Bruce, M.E., et al., *Strain characterization of natural sheep scrapie and comparison with BSE.* J Gen Virol, 2002. **83**(Pt 3): p. 695-704.
108. Wadsworth, J.D., et al., *Human prion protein with valine 129 prevents expression of variant CJD phenotype.* Science, 2004. **306**(5702): p. 1793-6.
109. Sigurdson, C.J., et al., *Strain fidelity of chronic wasting disease upon murine adaptation.* J Virol, 2006. **80**(24): p. 12303-11.
110. Benestad, S.L., et al., *Atypical/Nor98 scrapie: properties of the agent, genetics, and epidemiology.* Vet Res, 2008. **39**(4): p. 19.
111. Bessen, R.A., et al., *Non-genetic propagation of strain-specific properties of scrapie prion protein.* Nature, 1995. **375**(6533): p. 698-700.
112. Vorberg, I. and S.A. Priola, *Molecular basis of scrapie strain glycoform variation.* J Biol Chem, 2002. **277**(39): p. 36775-81.
113. Polymenidou, M., et al., *Coexistence of multiple PrPSc types in individuals with Creutzfeldt-Jakob disease.* Lancet Neurol, 2005. **4**(12): p. 805-14.
114. Manuelidis, L., *Vaccination with an attenuated Creutzfeldt-Jakob disease strain prevents expression of a virulent agent.* Proc Natl Acad Sci U S A, 1998. **95**(5): p. 2520-5.
115. Bartz, J.C., et al., *Prion interference is due to a reduction in strain-specific PrPSc levels.* J Virol, 2007. **81**(2): p. 689-97.
116. Aguzzi, A., *Protein conformation dictates prion strain.* Nat Med, 1998. **4**(10): p. 1125-6.
117. Weissman, J.S. and J.K. Hood, *Prion diseases. A rogue protein.* Lancet, 2001. **358 Suppl**: p. S53.
118. Scott, M., et al., *Transgenic mice expressing hamster prion protein produce species-specific scrapie infectivity and amyloid plaques.* Cell, 1989. **59**(5): p. 847-57.

119. Kimberlin, R.H. and C.A. Walker, *Evidence that the transmission of one source of scrapie agent to hamsters involves separation of agent strains from a mixture.* J Gen Virol, 1978. **39**(3): p. 487-96.
120. Wuthrich, K. and R. Riek, *Three-dimensional structures of prion proteins.* Adv Protein Chem, 2001. **57**: p. 55-82.
121. Goldmann, W., et al., *Two alleles of a neural protein gene linked to scrapie in sheep.* Proc Natl Acad Sci U S A, 1990. **87**(7): p. 2476-80.
122. Goldmann, W., *PrP genetics in ruminant transmissible spongiform encephalopathies.* Vet Res, 2008. **39**(4): p. 30.
123. Belt, P.B., et al., *Identification of five allelic variants of the sheep PrP gene and their association with natural scrapie.* J Gen Virol, 1995. **76 (Pt 3)**: p. 509-17.
124. Elsen, J.M., et al., *Genetic susceptibility and transmission factors in scrapie: detailed analysis of an epidemic in a closed flock of Romanov.* Arch Virol, 1999. **144**(3): p. 431-45.
125. Buschmann, A., et al., *Atypical scrapie cases in Germany and France are identified by discrepant reaction patterns in BSE rapid tests.* J Virol Methods, 2004. **117**(1): p. 27-36.
126. Moum, T., et al., *Polymorphisms at codons 141 and 154 in the ovine prion protein gene are associated with scrapie Nor98 cases.* J Gen Virol, 2005. **86**(Pt 1): p. 231-5.
127. Orge, L., et al., *Identification of putative atypical scrapie in sheep in Portugal.* J Gen Virol, 2004. **85**(Pt 11): p. 3487-91.
128. Beringue, V., J.L. Vilotte, and H. Laude, *Prion agent diversity and species barrier.* Vet Res, 2008. **39**(4): p. 47.
129. Bueler, H., et al., *Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein.* Nature, 1992. **356**(6370): p. 577-82.
130. Brandner, S., et al., *Identification of the end stage of scrapie using infected neural grafts.* Brain Pathol, 1998. **8**(1): p. 19-27.
131. Brandner, S., et al., *Normal host prion protein necessary for scrapie-induced neurotoxicity.* Nature, 1996. **379**(6563): p. 339-43.
132. Lucassen, P.J., et al., *Detection of apoptosis in murine scrapie.* Neurosci Lett, 1995. **198**(3): p. 185-8.
133. Giese, A., et al., *Neuronal cell death in scrapie-infected mice is due to apoptosis.* Brain Pathol, 1995. **5**(3): p. 213-21.
134. Jesionek-Kupnicka, D., et al., *Apoptosis in relation to neuronal loss in experimental Creutzfeldt-Jakob disease in mice.* Acta Neurobiol Exp (Wars), 2001. **61**(1): p. 13-9.
135. Dorandeu, A., et al., *Neuronal apoptosis in fatal familial insomnia.* Brain Pathol, 1998. **8**(3): p. 531-7.
136. Gray, F., et al., *[Neuronal apoptosis in human prion diseases].* Bull Acad Natl Med, 1999. **183**(2): p. 305-20; discussion 320-1.
137. Gray, F., et al., *Neuronal apoptosis in Creutzfeldt-Jakob disease.* J Neuropathol Exp Neurol, 1999. **58**(4): p. 321-8.
138. Theil, D., et al., *Nuclear DNA fragmentation and immune reactivity in bovine spongiform encephalopathy.* J Comp Pathol, 1999. **121**(4): p. 357-67.

139. Chretien, F., et al., *[A process of programmed cell death as a mechanisms of neuronal death in prion diseases]*. Clin Exp Pathol, 1999. **47**(3-4): p. 181-91.
140. Jesionek-Kupnicka, D., et al., *Programmed cell death (apoptosis) in Alzheimer's disease and Creutzfeldt-Jakob disease.* Folia Neuropathol, 1997. **35**(4): p. 233-5.
141. Kuwahara, C., et al., *Prions prevent neuronal cell-line death.* Nature, 1999. **400**(6741): p. 225-6.
142. Yin, X.M., Z.N. Oltvai, and S.J. Korsmeyer, *BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax.* Nature, 1994. **369**(6478): p. 321-3.
143. Adams, J.M. and S. Cory, *The Bcl-2 protein family: arbiters of cell survival.* Science, 1998. **281**(5381): p. 1322-6.
144. Kurschner, C. and J.I. Morgan, *Analysis of interaction sites in homo- and heteromeric complexes containing Bcl-2 family members and the cellular prion protein.* Brain Res Mol Brain Res, 1996. **37**(1-2): p. 249-58.
145. Kurschner, C. and J.I. Morgan, *The cellular prion protein (PrP) selectively binds to Bcl-2 in the yeast two-hybrid system.* Brain Res Mol Brain Res, 1995. **30**(1): p. 165-8.
146. Borchelt, D.R., et al., *Scrapie and cellular prion proteins differ in their kinetics of synthesis and topology in cultured cells.* J Cell Biol, 1990. **110**(3): p. 743-52.
147. DebBurman, S.K., et al., *Chaperone-supervised conversion of prion protein to its protease-resistant form.* Proc Natl Acad Sci U S A, 1997. **94**(25): p. 13938-43.
148. Chabry, J., et al., *Species-independent inhibition of abnormal prion protein (PrP) formation by a peptide containing a conserved PrP sequence.* J Virol, 1999. **73**(8): p. 6245-50.
149. Hill, A.F., M. Antoniou, and J. Collinge, *Protease-resistant prion protein produced in vitro lacks detectable infectivity.* J Gen Virol, 1999. **80 (Pt 1)**: p. 11-4.
150. Forloni, G., et al., *Neurotoxicity of a prion protein fragment.* Nature, 1993. **362**(6420): p. 543-6.
151. Supattapone, S., et al., *Prion protein of 106 residues creates an artifical transmission barrier for prion replication in transgenic mice.* Cell, 1999. **96**(6): p. 869-78.
152. Supattapone, S., et al., *A protease-resistant 61-residue prion peptide causes neurodegeneration in transgenic mice.* Mol Cell Biol, 2001. **21**(7): p. 2608-16.
153. Radovanovic, I., et al., *Truncated prion protein and Doppel are myelinotoxic in the absence of oligodendrocytic PrPC.* J Neurosci, 2005. **25**(19): p. 4879-88.
154. Shmerling, D., et al., *Expression of amino-terminally truncated PrP in the mouse leading to ataxia and specific cerebellar lesions.* Cell, 1998. **93**(2): p. 203-14.
155. Stahl, N., et al., *Scrapie prion protein contains a phosphatidylinositol glycolipid.* Cell, 1987. **51**(2): p. 229-40.
156. Hay, B., et al., *Biogenesis and transmembrane orientation of the cellular isoform of the scrapie prion protein [published errratum appears in Mol Cell Biol 1987 May;7(5):2035]*. Mol Cell Biol, 1987. **7**(2): p. 914-20.
157. Hegde, R.S., et al., *A transmembrane form of the prion protein in neurodegenerative disease.* Science, 1998. **279**(5352): p. 827-34.

158. Hegde, R.S., S. Voigt, and V.R. Lingappa, *Regulation of protein topology by trans-acting factors at the endoplasmic reticulum.* Mol Cell, 1998. **2**(1): p. 85-91.

CHAPTER 2: Y145Stop is sufficient to induce de novo generation of prions using protein misfolding cyclic amplification

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Ahmed Abdallah, Ping Wang, Juergen A. Richt, and Srinand Sreevatsan

A point mutation in *Prnp* that converts tyrosine (Y) at position 145 into a stop codon leading to a truncated prion molecule as found in an inherited transmissible spongiform encephalopathy (TSE), Gerstmann-Sträussler-Scheinker syndrome, suggests that the N-terminus of the molecule (spanning amino acids 23-144) likely plays a critical role in prion misfolding as well as in protein-protein interactions. We hypothesized that Y145Stop molecule represents an unstable part of the prion protein that is prone to spontaneous misfolding. Utilizing protein misfolding cyclic amplification (PMCA) we show that the recombinant polypeptide corresponding to the Y145Stop of sheep and deer PRNP can be in vitro converted to PK-resistant PrP^{Sc} in presence or absence of preexisting prions. In contrast, recombinant protein full-length PrP^{C} did not show a propensity for spontaneous conformational conversion to protease resistant isoforms. Further, we show that seeded or spontaneously misfolded Y145Stop molecules can efficiently convert purified mammalian PrP^{C} into protease resistant isoforms. These results establish that the N-terminus of PrP^{C} molecule corresponding to residues 23-144 plays a role in seeding and misfolding of mammalian prions.

Introduction

Transmissible spongiform encephalopathies (TSEs) are a group of prion diseases that include Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI) in humans, as well as scrapie in sheep, chronic wasting disease (CWD) in deer, and bovine spongiform encephalopathy (BSE) in cattle[1-3]. A critical aspect of the pathogenesis of TSEs involves conformational conversions of normal cellular prion protein (PrP^{C}) to an abnormal isoform, termed PrP^{Sc}[4]. Although it is currently unknown whether PrP^{Sc} from subclinical or preclinical animals of a variety of species can be present in conformations (or "strains") that can cause human or animal TSEs, experimental studies have established that interspecies transmission of chronic wasting disease (CWD), bovine spongiform encephalopathy (BSE), and scrapie can occur under laboratory conditions. Further evidence supporting interspecies transmission is derived from the recent demonstration that the prion strain causing BSE is indistinguishable from the strain causing vCJD. Both share common incubation periods and lesion profiles in the brains of infected mice, macaques, and they have identical glycosylation profiles. Available data in the literature strongly suggest that conformations of PrP^{Sc} are critical for the interspecies transmission of TSE. Primary amino acid sequences of PRNP as well as the conformation of the infectious seeding prion appear to be important in defining strains and their potential transmissibility.

In addition to the acquired infections, over 20 mutations in human *Prnp* have been shown to be associated with prion phenotypes in humans[2]. The mutations are hypothesized to destabilize the mutant PRNP, which then undergoes a spontaneous conformational change into the protease-resistant and pathogenic form. A point mutation

in *Prnp* that converts tyrosine (Y) at position 145 into a stop codon leading to a truncated prion molecule, present in an autosomal dominant inherited genetic TSE, called Gerstmann-Sträussler-Scheinker syndrome, suggests that the N-terminus of the molecule (spanning amino acids 23-144) plays a critical role in prion misfolding as well as in protein-protein interactions. The N-terminus of the prion protein is largely unstructured and does not contain stable secondary structures [5] and is highly conserved across different species. This disease-related mutation suggests that the N-terminus of the molecule (PrP23-144) plays a critical role in prion misfolding as well as in protein-protein interactions. There is lack of detailed structural information regarding the conversion process of prion protein as well as PrP^{Sc} and its many possible conformers. In the present study, the self-propagating conversion of the purified recombinant protein was demonstrated with peptides corresponding to PrP145Stop from sheep and deer. Furthermore, we compared the efficiency with which spontaneously converted PrP145Stop induced conversion of recombinant and mammalian PrP^{C}.

Results

Cloning and expression of the Recombinant PrP145Stop

Open reading frame encoding Y145Stop of sheep and deer encompassing residues 23-144 of human prion protein (huPrP23-144) was cloned and expressed in *E. coli* and purified based on the affinity of the conserved octapeptide repeats for transition-metal cations. All expression plasmids were verified for sequence orientation and accuracy by DNA sequencing and the amino acid sequences of known deer and sheep sequences were compared by clustalW (Figure 1). Purified Y145Stop from different species were seen on Western blot using a prion specific monoclonal antibody (1E4; Fitzgerald Industries International, Concord, MA, USA) targeting an epitope spanning amino acids 108-119 before and after PK digestion (Figure 2A).

Spontaneous Conversion of the Recombinant PrP145Stop of sheep and deer

Freshly purified proteins were dialyzed against 10mM sodium phosphate, pH 6.5 overnight. After prolonged periods of storage at room temperature, the proteins remained in a monomeric form with no sign of self-association as verified by the ThT assay. To test the conformational conversion of PrP145Stop, we performed two rounds of PMCA using the recombinant prion polypeptides of sheep and deer encompassing residues 23 to144 (PrP23-144). In the first round, the reactions were incubated without addition of any seed. The subsequent round was seeded with a 1/10 volume of PMCA product from the previous round. Western blot analysis revealed that monomeric PrP145Stop without PMCA incubation was fully degraded when incubated for 1-hour in the presence of 1 μg/ml of proteinase K (Figure 2B). However, in the PMCA reactions, spontaneously

generated fibrillar proteins persisted after proteinase K digestion. This conversion was consistent for the purified recombinant Y145Stop of both deer and sheep (Fig. 1B). We next examined the conformational conversion of the purified recombinant full-length prion protein of sheep and deer strains; white-tailed deer (G96 and S96 variants) and sheep (171R). In contrast to PrP145Stop, none of the purified recombinant protein corresponding to full-length protein showed a propensity for spontaneous conversion to protease resistant isoforms (Figure 2C). To probe the progression of amyloid fibril formation, we used the fluorimetric ThT assay. A time course increase in ThT fluorescence was identified, suggesting that PrP145Stop from sheep and deer acquired the ability to bind ThT (Figure 3). The kinetic curves for the fluorescent uptake were characterized by a lag phase ≈ 10 h (Figure 3 A and B). Overall, the above data indicate that the conformational conversion of sheep and deer PrP23-144 (Y145Stop) is a self-seeded reaction.

Seeded Conversion of the Recombinant Y145Stop by PrP^{Sc} and PrP^{CWD}

To test if in-vitro amplification of protease resistant PrP145Stop proceeds in a seeded fashion, we next applied the PMCA using PrP^{Sc} and PrP^{CWD}, as seeds, from infected and confirmed brain tissues of sheep and deer, respectively. First, the minute amounts of brain-derived PrP^{Sc} (Scrapie) and PrP^{CWD} (Chronic wasting disease; CWD) were PMCA amplified using PrP^{C} from respective species. Purified recombinant Y145Stop of sheep and deer were also spiked with partially purified PrP^{Sc} from deer and sheep PMCA amplification in the presence of small amounts (1% wt/wt) of the deer and sheep PrP^{Sc} resulted in elimination of the lag phase (Figure 4 A, B). Similar conversion rates were

observed with the cross seeding between Y145Stop of the two species. Unlike PrP145Stop prion protein, the recombinant polypeptide corresponding to full-length prion protein (PrP 23-231) of sheep and deer did not show efficient amplification when the same molecules as above were used as seeding agents.

Seeded Conversion of mammalian PrP^{C} by prion-seeded or spontaneously generated PrP^{Res} of Y145Stop molecules

The efficiency of seeding between proteins derived from sheep and deer was examined next. A second passage experiment was performed with purified mammalian (deer and sheep) PrP^{C} as templates. Unseeded control PMCA experiments showed no spontaneous conversion for any mammalian PrP^{C} tested. Electrophoretic mobility patterns of seeded misfolded sheep PrP^{C} was identical regardless of whether the seed was PrP^{Sc}, PrP^{CWD}, or any misfolded Y145Stop (Figure 5). Identical results were obtained when PrP^{C} from deer was used as a template for PMCA (data not shown). Further, the results suggest that in-vitro converted prions of deer and sheep carry similar protease cleavage sites and glycosylation profiles do not change during the process of PMCA.

Discussion

The conversion of PrP^{C} isoform into the PrP^{Sc} isoform may occur spontaneously or sporadically, be genetic as in familial prion diseases or be acquired exogenously via consumption or iatrogenic routes as in infectious prion diseases. Conversion of the prion protein from the cellular to pathological isoform is central to the pathogenesis of prion disorders. The mechanism of this conformational conversion remains poorly understood and the critical part of the molecule involved in catalysis of misfolding is not known. To provide a better understanding of prion misfolding, we investigated the role of the N-terminus of PRNP in conversion. The Y145Stop mutation at PRNP is the basis of a naturally occurring familial spongiform encephalopathy; Gerstmann-Sträussler-Scheinker disease (GSS). Elucidation of how the Y145Stop molecule contributes to the mechanism of conformational conversion would provide insight into the underlying pathogenesis and, indeed, the prion concept itself. We propose that Y145Stop is sufficient for prion misfolding and could also play a role in interspecies transmission.

Y145Stop variant from sheep and deer can spontaneously polymerizes to protease resistant isoforms

We focused on the N-terminal region of the prion protein as it is largely unstructured and lacks the C-terminal 146-231 amino acids including the glycophosphatidyl anchor, which links PrP to the cell surface and the two sites for N-glycosylation, which are known to stabilize the PrP molecule. Models of prion conversion have suggested that formation of PrP^{Sc} involves refolding of residues within the region between residues 90 and 140 into β–sheets [2, 6]. The region that contains the residues 113-128 is most highly

conserved among all species studied[7]. Our data demonstrate that the purified recombinant polypeptide corresponding to the Y145Stop variant of sheep and deer spontaneously polymerizes to β–sheets structures which bind Thioflavin T (ThT). The self-propagating conversion by PMCA not only occurs in HuPrPY145Stop [8-10], but also in the corresponding sheep and deer molecules. A similar conversion capacity among various species is likely due to the high percentage of amino acid sequence similarity in regions 113-128 and 138-141 in the N-terminal part of PrP^{C} [8]. The latter region has been reported to influence PrP^{Sc} formation [11-13] and may be essential for nucleating intermolecular interactions [8]. The conformational conversion of PrP145Stop is a nucleation-dependent reaction in which the growth of amyloid is preceded by a lengthy lag phase ranging from 8-10 hrs [9] which is characteristic for nucleation-dependent polymerization [8-10]. Many experimental evidences such as the infectious truncated HuPrP (PrP 27-30)[14], the shorter version PrP106 acting as barrier for prion replication[15], and the Japanese patient carrying the mutation Y145Stop [16] suggests a critical role for the segment MoPrP(89–143) in conversion. Tg196 mice expressing MoPrP(P101L) at low level, intracerebrally inoculated with the β-amyloid forms of MoPrP(89–143), exhibited neurological changes similar to those present in GSS patients [17] and transmitted the disease to other Tg196 mice suggesting the de novo GSS prion formation[18]. Similarly, Y145Stop of sheep and deer spontaneously misfold and the conserved sequence homology across species supports the idea that parts of the N-terminus of the prion molecule may be involved in prion conversion and may participate in crossing species barriers. More targeted studies are needed to confirm the role of Y145Stop in interspecies transmission. These studies on spontaneously misfolded

Y145Stop from multiple species and their role in interspecies transmission are underway in our laboratory.

Conversion of the recombinant PrP145Stop is accelerated by addition of preexisting seeds.

Autocatalytic conversion from PrP^{C} into PrP^{Sc} is a key feature of prion replication [19]. We demonstrated that in vitro conversion of PrPY145Stop exhibits attributes of the autocatalytic mechanism, such as lag phase and seeding phenomena. Importantly, addition of a small quantity of spontaneously converted Y145Stop from sheep or deer to soluble protein monomer was able to seed the conversion and eliminate the lag phase. Therefore, sheep and deer proteins were found to be fully compatible, i.e., ShPrP23-144 fibrils readily seeded deer protein and DePrP23-144 fibrils acted as efficient seed for fibrilization of sheep PrP23-144. Furthermore, similar self-assembly curves were observed with cross-seeding by preformed aggregates from scrapie- or deer-infected brain. Kundu et al. [8] found that the residues within the 138–141 region of PrP are important for amyloid formation. Interestingly, one of the mismatches between Hu and Syrian hamster PrP is located at position 139 (isoleucine in Hu PrP and methionine in sha PrP) which led to lack of cross seeding between the two species [9]. This region shares 100% amino acid similarity in sheep and deer PrP145Stop. Whether this or other positions within the region 90–231 influences the species specificity of autocatalytic conversion remains to be determined. The present data show that there is little effect of species-specific sequence variation in PrPY145Stop sequence on the efficiency of cross-

seeding between deer and sheep. These findings point to a role of the N-terminal unstructured segment for prion transmissibility between different species.

Full-length prion protein resists spontaneous conversion

The full-length PrP obviously cannot mimic all aspects of conversion of the N-terminal region of the prion protein, in which the C-terminal domain might protect the full molecule from triggering the conversion of PrP^{C} to PrP^{Sc} by shielding the critical amyloidogenic determinant(s) that is exposed in the unfolded N-terminal region of PrP [20, 21]. Similar studies with the recombinant full-length PrP were not possible due to practical difficulties in propagating efficient seeded conversion of full-length PrP, although certain aspects of seeded conversion of PrP23–231 or PrP90–231 could be reproduced using a reduction-oxidation process [20] or under conditions requiring high concentrations of denaturants and very vigorous shaking[21]. Recently, PMCA has been used to generate infectious prions from bacterially-expressed full-length murine [22] and Syrian hamster [23, 24] recombinant PrP. However, additional cofactors were required for efficient PrP^{Sc}-seeded PMCA reaction. In one study [22], the proteinase K (PK)–resistance was detected after 17 rounds of PMCA which could be explained as a conformational adaptation allowing the newly emerged conformation (strain) to cross species barriers and serially propagate.

Spontaneously converted PrP145Stop is sufficient to induce de novo generation of PrP^{Sc} from mammalian PrP^{C}

Next we asked if the spontaneously misfolded Y145Stop molecules were sufficient to seed mammalian prions. Amplification of TSE infectivity was also accomplished by PMCA by employing purified PrP^{C} as a substrate [22, 25], exclusively in presence of polyanions. The efficient conversion of Y145Stop raises the question as to whether this molecule could also convert PrP^{C} extracted from normal brain tissues. Our data suggest that protease resistant prions can indeed be generated *de novo* from mammalian PrP using spontaneously converted Y145Stop as a seed. The electrophoretic profiles of PrP^{Sc} from sheep protein inoculated with in vitro converted Y145Stop, as well as inoculated deer protein, were indistinguishable from those seeded with sheep (scrapie) and deer (chronic wasting disease; CWD), indicating similar PK cleavage site(s), as well as a similar ratio of different PrP^{Sc} glycoforms. The efficient conversion of mammalian prions of sheep and deer using spontaneously converted PrP145Stop indicates that PrP23-144 fibrils acted as an efficient seed for fibrillization of mammalian prions.

Several studies have shown that a variety of molecules, such as RNA [26],, DNA[27], and heavy metals including copper [28, 29] appear to stimulate prion conversion. The N-terminal fragment of PrP has been shown to bind with copper[30], DNA[31, 32], and RNA [33] whereas the C-terminal fragment (122-231) does not demonstrate a similar binding affinity to these molecules. On the basis of the results presented here, we propose a two-step phenomenon in prion misfolding. We propose that in the first instance, any one of these cofactors maybe involved in binding to and misfolding of the N-terminal region of the molecule, which then likely catalyzes

conversion of the full prion molecule. A detailed biochemical analyses and carefully designed studies that include PRNP structural variants would be needed to fully elucidate this model of prion conversion.

In summary, our findings demonstrate that the highly unstable part of the prion molecule, Y145Stop, can be converted in vitro to beta-sheet rich oligomeric amyloid fibrils in presence or absence of preexisting seed in sheep and deer species. Moreover, spontaneously converted PrP145Stop is sufficient to induce *de novo* generation of PrP^{Sc} from mammalian prions. It is possible that the nucleotide sequence similarity and the spontaneous nature of misfolding in N-terminal segment of the prion protein could also contribute to interspecies transmission.

Material and Methods

Plasmid construction

DNA was extracted from normal brain extracts of sheep and deer using standard protocols (Qiagen DNA extraction Kits, Valencia, CA). Y145Stop segments of *PrnP* were amplified using the forward primer as 5′-GGAATTCCATATGAAGAAGCGACCAAAACCTGG-3′ for both sheep and deer, while 5′-CCCAAGCTTGGGTTAGTCATTGCCAAAATGTATAA-3′ and 5′-CCCAAGCTTGGGTTAGTCGTTGCCAAAATGTATAA-3′ as the reverse primers for sheep and deer respectively. Open reading frames were subsequently cloned into the NdeI and HindIII (NEB) restriction sites of the pET-41a vector (Novagen). The primer sets for Sh-R171 and De- G/S 96 were 5′ GGGATCCATCATGAAGCGACCAAAACCTG-3′ as the forward primer and 5′-CGAATTCTTATGCCCCCCTTTGGTAATAAGCC-3′ or 5′-CGAATTCTTATGCCCCTCTTTGGTAATAAG-3′ as the reverse primers for Sh-R171 and De- G/S96 respectively. All constructs in the pET-41a were verified for accuracy of insert orientation by DNA sequencing and the amino acid sequences were compared by clustalW against human and cattle Y145Stop (Fig.1.).

Protein expression and purification

Cloning and expression steps were combined by transforming the plasmid constructs into the Acella™ chemically competent Cells (Edge BioSystems). IPTG (1mM) induction was used to allow for larger scale expression of Y145Stop of each of the different species. Y145Stop purification method was based on the affinity of the conserved

octapeptide repeats for transition-metal cations avoiding the use of a histidine tag. The cell pellets were lysed in 8 M Urea, 0.1 NaH2PO4, 0.01 M Tris.Cl; PH 8.0 for one hour at 4^{o}C followed by sonication. The prepared protein extracts were added onto HisPur Cobalt Resin (Pierce). The wash step with NaH2PO4/ Tris.HCl buffer was used to eliminate the urea. Y145Stop was eluted by 300 mM imidazole solution and dialyzed against 10 mM sodium phosphate, PH 6.5 overnight. Under the same conditions, we expressed and purified recombinant full-length prion protein sheep and deer; white-tailed deer (G96 and S96) and sheep R at codon 171.

Purification of normal and pathological isoforms of prion protein from brain tissues:

PrP^{C} and PrP^{Sc} were purified by sucrose density-gradient centrifugation and subjected to ultracentrifugation methods. Brain samples of sheep or deer confirmed negative or positive for TSEs by immunohistochemistry at the Minnesota Veterinary Diagnostic Laboratory were used for extraction of prion proteins. Brain tissue was sliced and rinsed with cold PBS to remove the residual blood. Two syringes connected with a Three-Way Stopcock with Male Luer Slip Adapter (Baxter Healthcare Corp, Deerfield, IL, USA) were used to prepare 10% w/v brain suspension using cold 10% sarcosine (TEND) that was supplemented with 1mM DTT and ethylenediaminetetraacetic acid (EDTA)-free Protease Inhibitors cocktail (Roche, Mannheim, Germany) at each step where it was needed. Aliquots of this suspension were homogenized then transferred on ice for one hour. The mixture was subjected to centrifugation at 3,000 ×*g* for 10 minutes to pellet of cellular debris. The supernatants were transferred to new Optiseal tubes (Beckman

Coulter, Fullerton, CA) and centrifuged through 1 M sucrose cushion for 2 h 30 min at 4°C at 100 000 ×*g*. This pellet consists of a microsomal/ synaptosomal membrane fraction containing PrP^{C}. Then the pellets were rinsed using 10% NaCl, 1% SB 3-14 (TEND+ PIs). DNAase I and RNAase treatment were used to remove nucleic acids from the aliquots. At this point, a portion of the material was digested with different concentrations (1, 5, 10 µg/ml) of proteinase K[34] enzyme. The suspension was then subjected to centrifugation at 125,755 ×*g* for 2 hours and pellets were rinsed using 0.5% SB 3-14/1X PBS and finally re-suspended in 1 ml 2% sarkosyl. The protein concentration was adjusted using bicinchonic acid assay (Pierce, Rockford, IL, USA) to 0.5-1 mg/ml. protein purity was assessed by silver staining of gels (Figure 6).

In vitro conversion of PrP^{C} to PrP^{Sc} using PMCA assay

Prions replicate in the body by inducing the misfolding of the normal prion protein. The PMCA assay was developed to mimic this process and was performed as described,[35] in 0.2 ml thin wall PCR tube strips as 80-µl solutions containing PBS with 0.05% (wt/vol) SDS and 0.05% Triton X-100. PK-resistant fragments seeded by PrP^{Sc} were generated with seed-to-substrate ratios of 1:100 (40 ng of PrP^{Sc}). The tube strips were placed in floating rack in the Sonicator (Misonix Model 4000, Farmingdale, New York) cup horn with water at 37°C and were subjected to repeated cycles of sonication. The samples were subjected to two rounds of PMCA reaction, each of them consisting of 24 cycles with 40 sec intermittent sonication at 100% power (16 min total sonication time), 59 min 20 sec incubations between each sonication. After the first round, an aliquot of the amplified samples was taken and diluted 10-fold into the conversion buffer containing

the monomeric protein (0.5 mg/ml) as substrate. A second round of 24 cycles of sonication and incubation as described above was repeated. Buffer, vector control and unseeded sonication were used to test the tendency of the samples for spontaneous misfolding and/or contamination.

Proteinase K resistance assay

15 µl of the reaction sample (1 µg of rPrP) was diluted in PBS with 0.1% SDS and digested with PK. Typically; the PK concentration to a final volume of 3 µg/ml was used for one hour at 37^{o}C. The reaction was stopped by using Pefabloc (Roche) to final concentration of 4mM. The samples were denaturized using 2x SDS-buffer containing 4M urea then subjected the samples to 4-20% SDS-PAGE.

Immunoblotting

Samples were separated in 4-20% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. Monoclonal antibody (1E4) targeting an epitope spanning amino acids 108-119 was added with a 1:1,000 dilution. Secondary antibody conjugated with chemiluminescent compounds was added with a 1:10,000 dilution. All blots were developed using Supersignal West Pico Chemiluminescent Substrate (Pierce).

Thioflavin T (ThT) Assay

The progression of amyloid fibril formation was followed by a fluorimetric ThT assay. A stock solution of ThT dye (1 mg/ml; 3.14 mM) was prepared in distilled, deionized water. Small aliquots of each sample were withdrawn every 2 hrs up to 24 hrs and diluted to a

final concentration of 4 μM in 50 mM Phosphate buffer, pH 6.5 containing 10 μM ThT. After 2 min incubation at room temperature, the fluorescence of ThT dye was measured by a Spectramax M2 plate reader (Molecular Devices, Sunnyvale, CA) set to at 482 nm by using the excitation wavelength of 450 nm. ThT fluorescence was normalized by (Fi-F0)/(Fmax-F0). Fi was the ThT intensity (fluorescence arbitrary unit) of samples, and F0 was the ThT background intensity. For the measurements of polymerization at different time points, Fmax was the maximum ThT intensity of samples at the end of PMCA cycle.

Figures

Figure 1: Amino acid sequence alignment of Y145Stop from different species is shown. The alignment was obtained using ClustalW of huY145Stop with the corresponding sequences in cattle, deer, and sheep genome.

Figure 1: Amino acid sequence alignment of Y145Stop from different species

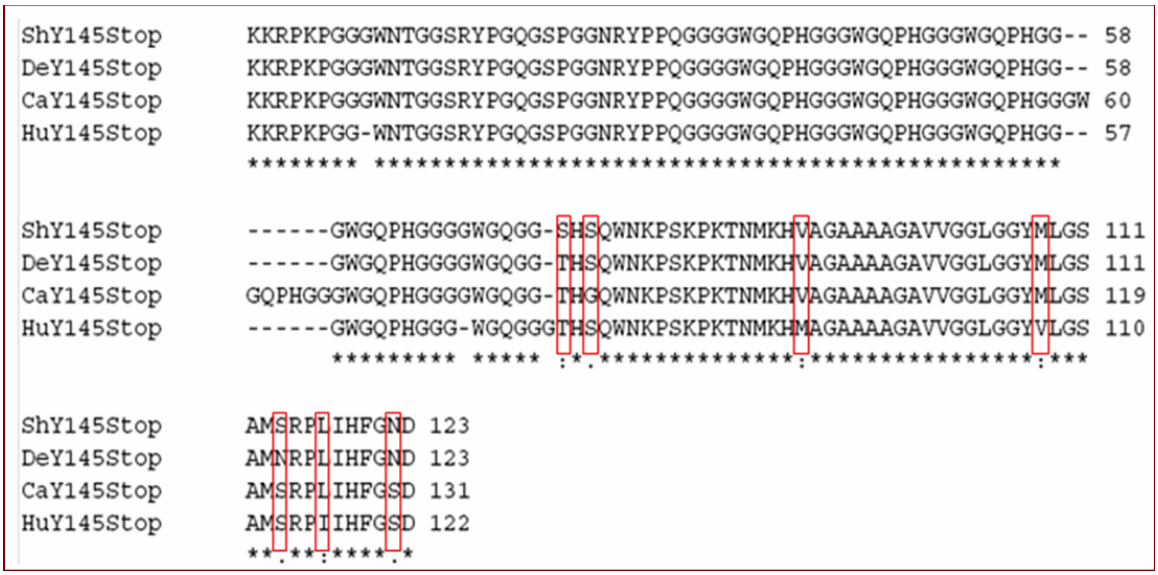


Figure 2: (A) Expression of full-length prion protein and Y145Stop of sheep and deer. Immunoblot of the purified proteins transfected with the expression vector alone or vectors containing constructs of the designated species (defined in the legend to the Figure 2.a.) shows full degradation when incubated for 1-hour in the presence of proteinase K. Positions of the molecular mass markers are designated in kDa.
B) Proteinase K digestion of sheep and deer Y145Stop molecules after PMCA at different time points. Human recombinant PrP 23-231 was used as a positive control. Spontaneously generated fibrillar proteins persisted after proteinase K digestion and were detected at 16 h with more conversion at 24 and 36 h after PMCA incubation.
(C) Immunoblotting of PrP-sen of the purified recombinant full-length prion protein of sheep and deer strains; white-tailed deer (G96 and S96 variants) and sheep (171R) after PMCA and PK digestion at different time points. Proteins were fully degraded in the presence of proteinase K with no detectable conversion after 72 h of PMCA.

Figure 2: Immunoblot of the purified proteins; before and after PMCA

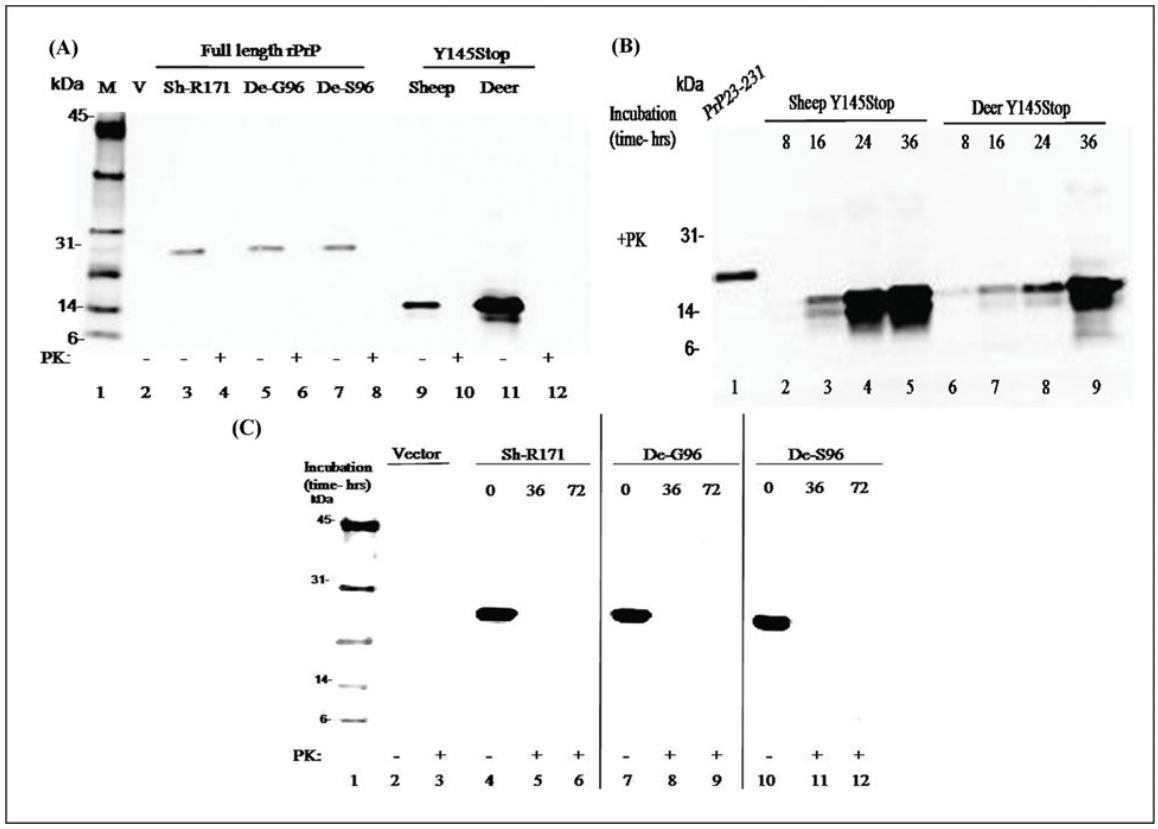


Figure 3: Time course analysis to document increase in Thioflavin T uptake and fluorescence by sheep and deer Y145Stop in the absence of seeds. (A) ShY145Stop (squares) and full length Sheep PrP with R at codon 171 (triangles) incubated in the absence of seeds. (B) DeY145Stop (circles) and full length Deer PrP with G (squares) or S (triangles) at codon 96 incubated with no seeds. All samples were subjected to PMCA under identical conditions. ShY145Stop and DeY145Stop acquired the ability to bind ThT without addition of external seeds. In contrast, the full-length prion protein did not show detectable ThT binding. The kinetic curves were characterized by a lag phase (8-12 hrs).

Figure 3: Time course analysis to document increase in Thioflavin T uptake in absence of seeds

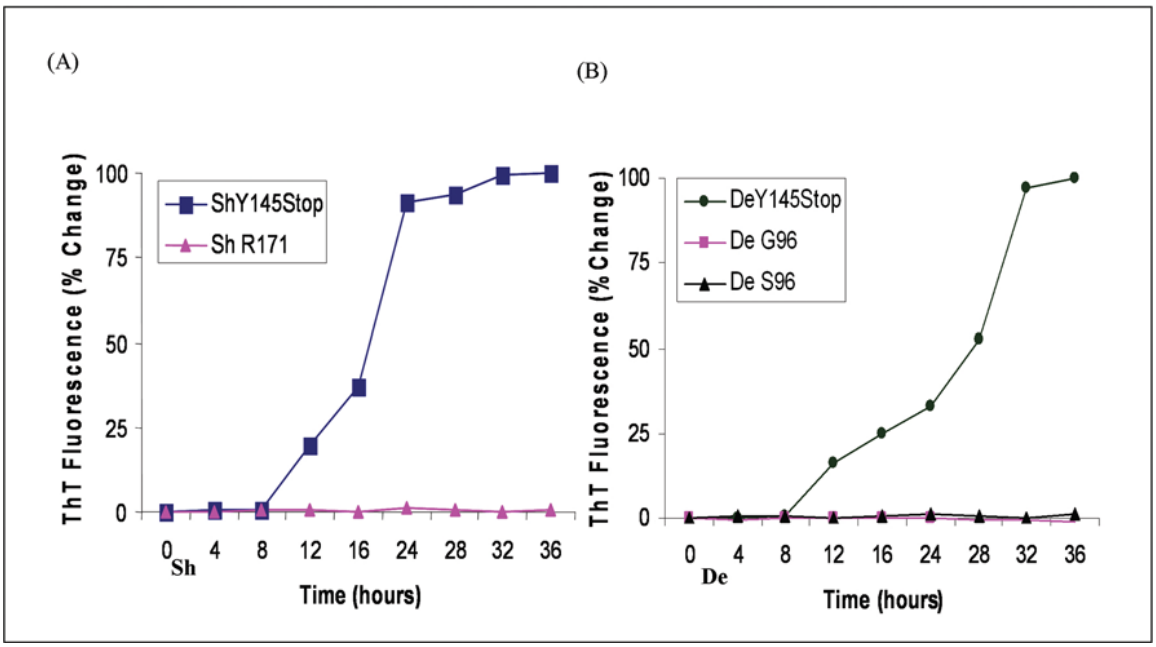


Figure 4: The progress of amyloid fibril formation measured by fluorimetric Thioflavin T uptake assay in presence of seeds. One percent (w/w) performed fibrillar seeds (denoted in square brackets) were added to solutions containing monomers of sheep (A) or deer (B) Y145Stop. No seed control showed the characteristic lag phase, while seeded reactions with partially purified PrP^{Sc} from deer [CWD] or sheep [Sc] eliminated the lag phase. Similar conversion rates were observed with cross seeding by in-vitro converted Y145Stop of sheep [ShY145Stop] or deer [DeY145Stop].

Figure 4: The progress of amyloid fibril formation measured by fluorimetric Thioflavin T uptake assay in presence of seeds

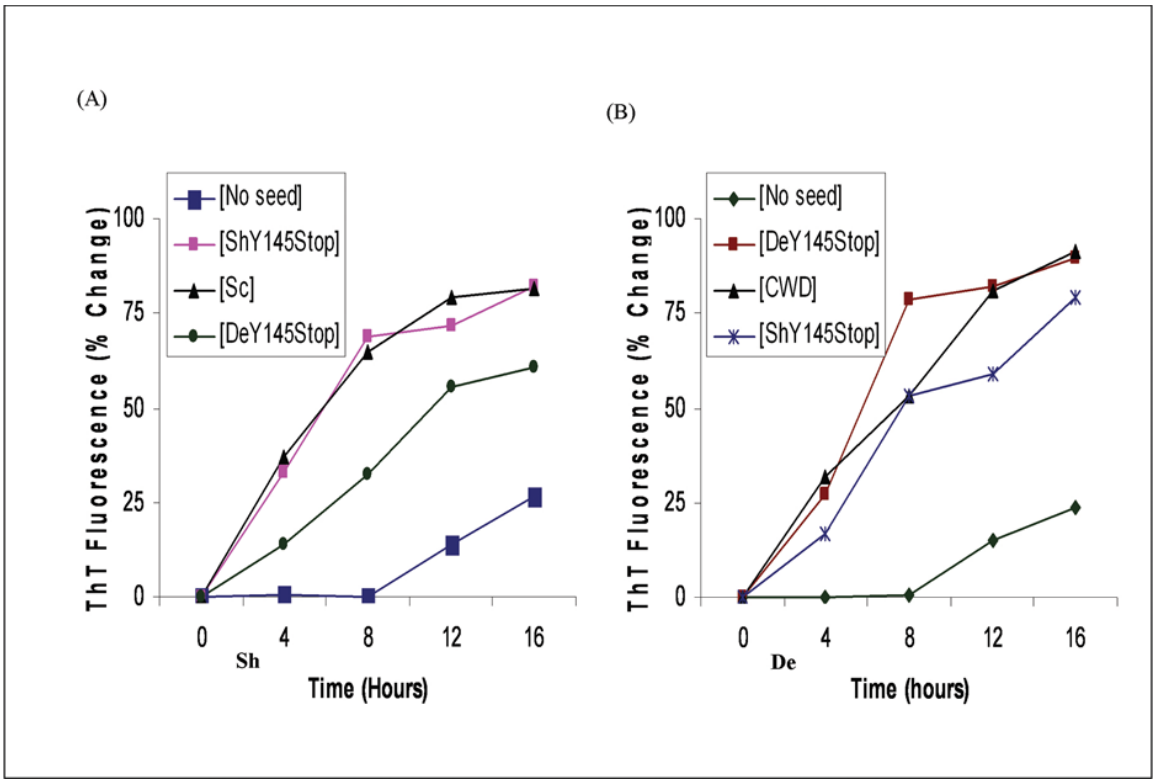


Figure 5: PMCA on purified PrP^{C} from brain tissue of sheep and deer. Lane 1, molecular weight marker (biotinylated). Lane 2, human recombinant PrP 23-231 was used as a positive control. Lanes 3 and 4, samples containing purified PrP^{C} from brain tissue of sheep before and after PK digestion. Lane 5, PrP^{Sc} extracted from sheep brain as a reference after PK digestion with 10 times the reaction equivalents of the seed protein concentration. Samples of purified PrP^{C} from brain tissue of sheep were incubated with preformed fibrillar seed of PrP^{Sc} round one (lane 6), round two (lane 7), PrP^{CWD} (lane 8), ShY145Stop seeded with performed fibrils of ShY145Stop (lane 10), DeY145Stop (lane 11), or PrP^{Sc} (lane 12) after PK digestion.

Figure 5: PMCA on purified PrP^C from brain tissue of sheep and deer

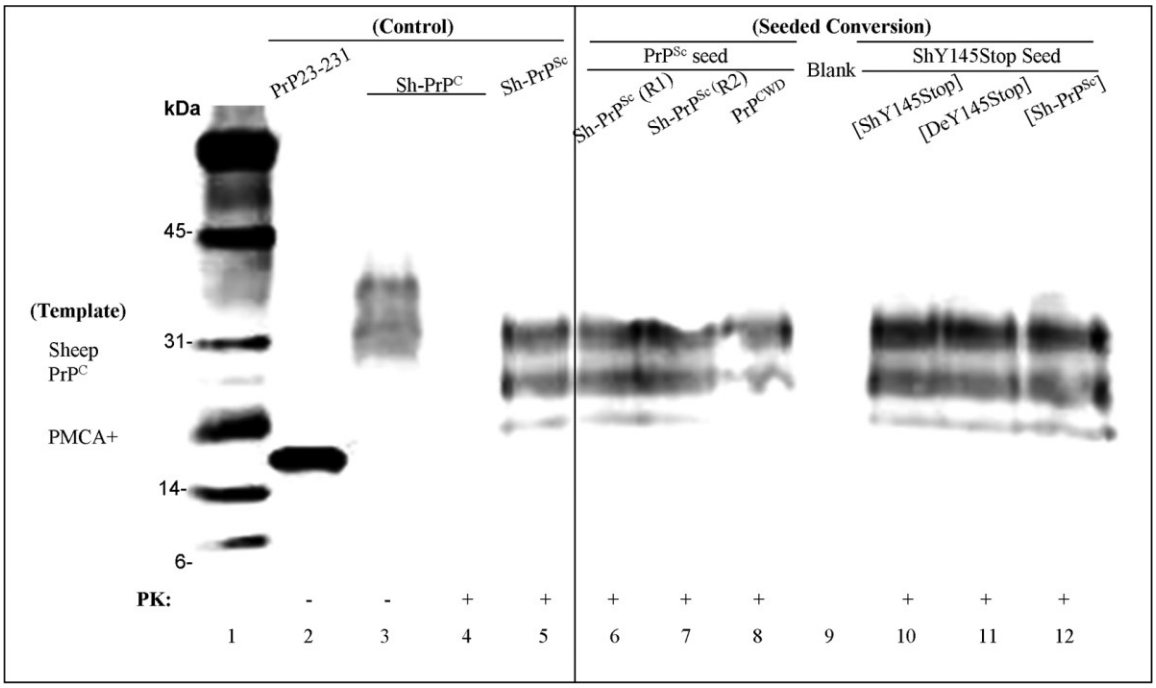
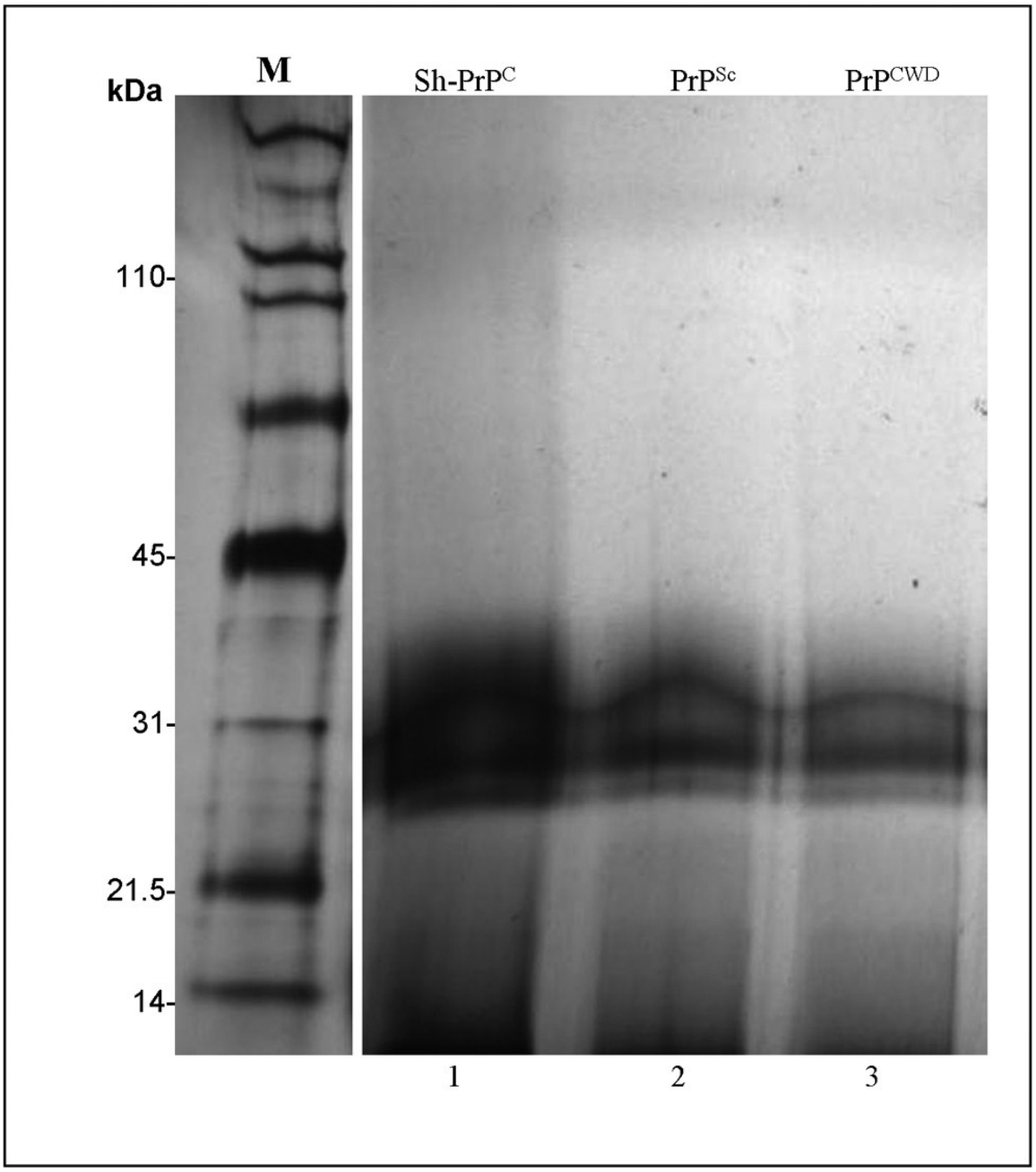


Figure 6: Silver Stain on purified prions from the brain tissues. Lane 1, purified PrPC derived from sheep brain. Lane2 and 3 purified PrPSc and PrPCWD extracted from infected brains tissues. All samples were resolved on 4-20% SDS-PAGE and stained with silver stain. A reference marker to the left is shown.

Figure 6: Silver Stain on purified prions from the brain tissues



References

1. Prusiner, S.B. and S.J. DeArmond, *Prion diseases and neurodegeneration.* Annu Rev Neurosci, 1994. **17**: p. 311-39.
2. Prusiner, S.B., *Prions.* Proc Natl Acad Sci U S A, 1998. **95**(23): p. 13363-83.
3. Collinge, J., *Prion diseases of humans and animals: their causes and molecular basis.* Annu Rev Neurosci, 2001. **24**: p. 519-50.
4. Prusiner, S.B., *Novel proteinaceous infectious particles cause scrapie.* Science, 1982. **216**(4542): p. 136-44.
5. Bruce, M., et al., *Transmission of bovine spongiform encephalopathy and scrapie to mice: strain variation and the species barrier.* Philos Trans R Soc Lond B Biol Sci, 1994. **343**(1306): p. 405-11.
6. Muramoto, T., et al., *Recombinant scrapie-like prion protein of 106 amino acids is soluble.* Proc Natl Acad Sci U S A, 1996. **93**(26): p. 15457-62.
7. Prusiner, S.B., *Prion diseases and the BSE crisis.* Science, 1997. **278**(5336): p. 245-51.
8. Kundu, B., et al., *Nucleation-dependent conformational conversion of the Y145Stop variant of human prion protein: structural clues for prion propagation.* Proc Natl Acad Sci U S A, 2003. **100**(21): p. 12069-74.
9. Vanik, D.L., K.A. Surewicz, and W.K. Surewicz, *Molecular basis of barriers for interspecies transmissibility of mammalian prions.* Mol Cell, 2004. **14**(1): p. 139-45.
10. Jones, E.M. and W.K. Surewicz, *Fibril conformation as the basis of species- and strain-dependent seeding specificity of mammalian prion amyloids.* Cell, 2005. **121**(1): p. 63-72.
11. Priola, S.A. and B. Chesebro, *A single hamster PrP amino acid blocks conversion to protease-resistant PrP in scrapie-infected mouse neuroblastoma cells.* J Virol, 1995. **69**(12): p. 7754-8.
12. Goldmann, W., et al., *Novel polymorphisms in the caprine PrP gene: a codon 142 mutation associated with scrapie incubation period.* J Gen Virol, 1996. **77 (Pt 11)**: p. 2885-91.
13. Zahn, R., et al., *NMR solution structure of the human prion protein.* Proc Natl Acad Sci U S A, 2000. **97**(1): p. 145-50.
14. Prusiner, S.B., et al., *Purification and structural studies of a major scrapie prion protein.* Cell, 1984. **38**(1): p. 127-34.
15. Supattapone, S., et al., *Prion protein of 106 residues creates an artifical transmission barrier for prion replication in transgenic mice.* Cell, 1999. **96**(6): p. 869-78.
16. Kitamoto, T., R. Iizuka, and J. Tateishi, *An amber mutation of prion protein in Gerstmann-Straussler syndrome with mutant PrP plaques.* Biochem Biophys Res Commun, 1993. **192**(2): p. 525-31.
17. Kaneko, K., et al., *A synthetic peptide initiates Gerstmann-Straussler-Scheinker (GSS) disease in transgenic mice.* J Mol Biol, 2000. **295**(4): p. 997-1007.
18. Tremblay, P., et al., *Mutant PrPSc conformers induced by a synthetic peptide and several prion strains.* J Virol, 2004. **78**(4): p. 2088-99.

19. Gajdusek, D.C., *Transmissible and non-transmissible amyloidoses: autocatalytic post-translational conversion of host precursor proteins to beta-pleated sheet configurations.* J Neuroimmunol, 1988. **20**(2-3): p. 95-110.
20. Lee, S. and D. Eisenberg, *Seeded conversion of recombinant prion protein to a disulfide-bonded oligomer by a reduction-oxidation process.* Nat Struct Biol, 2003. **10**(9): p. 725-30.
21. Baskakov, I.V., *Autocatalytic conversion of recombinant prion proteins displays a species barrier.* J Biol Chem, 2004. **279**(9): p. 7671-7.
22. Wang, F., et al., *Generating a prion with bacterially expressed recombinant prion protein.* Science, 2010. **327**(5969): p. 1132-5.
23. Makarava, N., et al., *Recombinant prion protein induces a new transmissible prion disease in wild-type animals.* Acta Neuropathol, 2010. **119**(2): p. 177-87.
24. Kim, J.I., et al., *Mammalian prions generated from bacterially expressed prion protein in the absence of any mammalian cofactors.* J Biol Chem, 2010. **285**(19): p. 14083-7.
25. Deleault, N.R., et al., *Formation of native prions from minimal components in vitro.* Proc Natl Acad Sci U S A, 2007. **104**(23): p. 9741-6.
26. Deleault, N.R., R.W. Lucassen, and S. Supattapone, *RNA molecules stimulate prion protein conversion.* Nature, 2003. **425**(6959): p. 717-20.
27. Cordeiro, Y., et al., *DNA converts cellular prion protein into the beta-sheet conformation and inhibits prion peptide aggregation.* J Biol Chem, 2001. **276**(52): p. 49400-9.
28. Quaglio, E., R. Chiesa, and D.A. Harris, *Copper converts the cellular prion protein into a protease-resistant species that is distinct from the scrapie isoform.* J Biol Chem, 2001. **276**(14): p. 11432-8.
29. Leclerc, E., et al., *Copper induces conformational changes in the N-terminal part of cell-surface PrPC.* Arch Virol, 2006. **151**(11): p. 2103-9.
30. Viles, J.H., et al., *Copper binding to the prion protein: structural implications of four identical cooperative binding sites.* Proc Natl Acad Sci U S A, 1999. **96**(5): p. 2042-7.
31. Nandi, P.K., *Interaction of prion peptide HuPrP106-126 with nucleic acid.* Arch Virol, 1997. **142**(12): p. 2537-45.
32. Takemura, K., et al., *DNA aptamers that bind to PrP(C) and not PrP(Sc) show sequence and structure specificity.* Exp Biol Med (Maywood), 2006. **231**(2): p. 204-14.
33. Gabus, C., et al., *The prion protein has RNA binding and chaperoning properties characteristic of nucleocapsid protein NCP7 of HIV-1.* J Biol Chem, 2001. **276**(22): p. 19301-9.
34. Raymond, G.J., Bossers A, Raymond LD, O'Rourke KI, McHolland LE, Bryant PK 3rd, Miller MW, Williams ES, Smits M, Caughey B., *Evidence of a molecular barrier limiting susceptibility of humans, cattle and sheep to chronic wasting disease.* EMBO J. , 2000 **19** (17): p. 4425-30. .
35. Atarashi R, M.R., Sim VL, Hughson AG, Dorward DW, Onwubiko HA, Priola SA, Caughey B., *Ultrasensitive detection of scrapie prion protein using seeded conversion of recombinant prion protein.*
. Nat Methods, 2007. **4**(8): p.:645-50.

CHAPTER 3: Prion seeding activity of misfolded N-terminal of prion protein in cell culture

Prion pathogenesis involves conformational conversion of the cellular prion protein (PrP^{C}) into the pathologic isoform (PrP^{Sc}). We have shown that recombinant prion protein variant Y145Stop (PrP23-144) can induce in vitro generation of PK-resistant PrP^{Sc} (PrP^{res}). We hypothesized that in-vitro converted C-terminally truncated prions would be infectious and lead to PrP^{res} amplification in cells. We tested this hypothesis using an ex-vivo Mule Deer Brain (MDB), immortalized cell line transfected with a plasmid containing the simian virus 40 genome, which expresses PrP^{C}. The cells were inoculated with the in-vitro converted PrP variants and the conversion of PrP^{C} was indexed. CWD-associated, MDB^{CWD} cells, protease-resistant prion protein (PrP^{CWD}) were used as positive controls. In-vitro converted Y145Stop triggered accumulation of protease-resistant prion protein MDB cells. Unlike Y145Stop variants, recombinant protein full-length PrP^{C} and N-terminal truncated prion molecules (90-231 and 146-231) did not recapitulate this process. This suggests that the seeding and, possibly, protein interacting sites exist in the N-terminus of the PrP^{C} molecule.

INTRODUCTION

A defining biochemical characteristic of prion diseases is the conformational conversion of PrP^{C} into PrP^{Sc}, an insoluble and partially protease-resistant isoform that propagates itself by imposing its abnormal conformation onto PrP^{C} molecules [1]. PrP^{C} is monomeric and rich in α-helices (42%) with a small percentage of β-sheets (3%) [2]. Upon conversion into PrP^{Sc}, β-sheet content increases to 43% versus 30% of the α-helical structure [2, 3]. PrP^{Sc} is believed to self-perpetuate by a interacting with PrP^{C} and catalyzing its conversion to the PrP^{Sc} state [4]. Despite recent progress in the infectious aspects of prions, the cellular mechanism of PrP misfolding and formation of an intermediate complex remains unclear. It has been hypothesized that PrP^{C} may bind to PrP^{Sc}, possibly in combination with auxiliary proteins, to form an intermediate complex during the formation of de novo PrP^{Sc} [5-7]. Studies have shown that TSE infectivity was accomplished by protein misfolding cyclic amplification (PMCA), a technique by which PrP^{Sc} is amplified by incubation with large amount of the normal brain homogenate [8, 9]. The system consists on cycles of accelerated prion replication to reach an exponential increase in the conversion. Recently, amplification of TSE infectivity was also accomplished by PMCA by employing purified PrP^{C} as a substrate [10, 11], exclusively in presence of polyanions. These data suggest that additional cofactors may be required to facilitate the conversion of the recombinant forms of PrP.

While the sporadic form is the most common among prion diseases (85%), the autosomal dominant inherited PrP gene (*Prnp*) mutations cause approximately 10% of all prion diseases [12]. In the inherited prion diseases, mutations in *Prnp* are hypothesized to favor spontaneous formation of PrP^{Sc} leading to neurodegeneration. Gerstmann-

Sträussler-Scheinker syndrome (GSS), an autosomal dominant prion disease, is associated with eight distinct single point mutations [13-15]. The autosomal dominant nature of this inheritance suggests that one mutant allele with, for example a Y145Stop substitution, is able to recruit and seed the 'other' allele [16]. While the mutant proteins are expressed thought the lifespan of the individual, disease rarely develops before the fourth of fifth decade of life, indicating slow progression of the disease [12]. Recently, we have shown that a GSS-associated prion protein variant Y145Stop (PrP23-144) can catalyze the conversion of mammalian PrP^{C} in-vitro using PMCA [17]. This study was performed with purified recombinant PrP23-144 and full-length PrP^{C} of deer and sheep (PrP23-231). This prompted us to investigate if these variants can trigger the conversion of PrP^{C} in a cell culture model. Several cell lines can be infected by prions as evidenced by the persistent accumulation of PrP^{Sc}, including the SMB [18], N2a [19, 20], GT1 [21], Rov9 [22], and fibroblast [23] cell lines. Recently, a mule deer brain-derived cell line (MDB) that expressed PrP^{C} and showed susceptibility to TSE infections, was developed [24]. This cell line was used to develop, MDB^{CWD}, a cell line that maintained stable PrP^{CWD} production through 32 serial passes [24]. In the present study, we used these cell lines to evaluate the infectious properties of in-vitro converted C-terminally truncated prions in ex vivo model.

RESULTS

C-terminus resists the conformational conversion into PrP-res by PMCA.

It has been addressed the propensity of C-terminals for spontaneous and seeded conversion into protease resistant isoforms. Open reading frame encoding C-terminals of deer encompassing residues 90-231 and 146-231 of human prion protein was expressed in *E. coli* and purified to homogeneity. Using seeding and PMCA protocols as performed on the Y145Stop [17], it has been examined the conformational conversion of the purified recombinant corresponding to C-terminal parts of the deer protein. Unlike PrP145Stop prion protein, none of the purified recombinant C-terminal regions showed detectable conformational conversion or a propensity to seed purified mammalian PrP^{C} into protease resistant isoforms (Figure 7).

Cell infectivity studies using misfolded Y145Stop

To assess the susceptibility of the MDB cell line to Y145Stop, cells were challenged with PMCA-misfolded Y145Stop from sheep or deer (Figure 8). Cells inoculated with purified PrP^{C} from brain and PMCA assay buffer served as negative controls. The *de novo* infection was assayed by cell blotting. As a positive control, MDB^{CWD} cell lysates or purified PrP^{CWD} from brain tissue were used for inoculation. Typically, cells were grown in the presence of the inoculum for 4 days before being split at a 1:10 ratio. Thereafter, cells were grown in uninoculated medium. The subcultures were passaged in parallel to minimize differences due to culture conditions. After 20 to 24 days in culture, the sublines were assayed by cell blotting. To ensure that only PK-

resistant PrP (PrP^{Sc})-expressing cells could be detected, the proteinase K (PK) concentration was optimized to ensure complete digestion of PrP^{C} (data not shown).

Twenty four days after inoculation, it has been compared the infectivity of the treatments to the CWD-infected cells (MDB^{CWD}). None of the negative control inocula showed detectable PrP^{Sc} in MDB cells (Figure 9A), while cell lysates of the MDB^{CWD} cells produced as much PrP^{Sc} as cloned MDB^{CWD}. PMCA misfolded Y145Stop induced prion conversion in 40% of the sublines, producing *de novo* infection as assayed by cell blotting (Figure 9B). Infected susceptible sublines with partially purified PrP^{CWD} behaved similarly. To confirm the presence of *de novo* PrP^{res} in the infected cell lines, Western blots were performed on PK-digested lysates of MDB cells challenged with Misfolded Y145Stop that showed infectivity by cell blotting. The presence of PrP^{res} in the cell lysate correlates with the *de novo* infection results obtained for Y145Stop in cell blotting (Figure 9C).

Kinetics of PrP^{Sc} production

It has been used a time course approach to study de novo PrP^{Sc} production in treated susceptible sublines (Figure 10). After 2 passages, 8 days post inoculation, weak signal of proteinase K-resistance was detected, which could be residue of the inoculum. Beginning the third passage postinoculation and thereafter, susceptible subclones produced detectable PrP^{Sc}. After six passages, the detectable amount of PrP^{Sc} in the inoculated cultures with misfolded Y145Stop approached that of PrP^{CWD} from brain tissue. Cultures negative at 8 days remained negative after 6 passages.

Comparison of the infectivity of Y145Stop- and MDB^{CWD} cell lysates-infected subclones

As shown previously in our study [17], there was little effect of species-specific sequence variation in PrP145Stop sequence on the efficiency of cross-seeding between deer and sheep PrP^{C}. To test whether this seeding characteristic correlated with the infectivity in our cell model, it has been used misfolded sheep Y145Stop (ShY145Stop) to inoculate the susceptible cell lines. A similar seeding characteristic was observed for misfolded DeY145Stop, ShY145Stop, and partially purified PrP^{CWD} (Figure 11). Again, it has been found that ~40% of the subclones were susceptible to ShY145Stop inocula. Nevertheless, benign MDB cells were more sensitive to MDB^{CWD} cell lysates than the misfolded Y145Stop inocula. Cell blots of inoculated cells with MDB^{CWD} cell lysates showed as much PrP^{Sc} as traditionally cloned MDB^{CWD}. Furthermore, 80% of the subclones were susceptible to treatments with respective proteins.

DISCUSSION

In this study, it has been demonstrated propagation of prions in cultured cells inoculated with PMCA-converted recombinant Y145Stop. The ability of misfolded Y145Stop to induce PrP^{Sc} formation in cell culture is comparable to that of prions extracted from infected brain tissues. The data suggest that once the N-terminal acquires spontaneous conformational conversion to protease resistant isoform, the prion cellular amplification is likely to be triggered.

The prion hypothesis postulates that the PrP^{Sc} isoform seeds the conversion of normal PrP^{C} to the pathogenic PrP^{Sc} by a self-propagating property. The propagation of prions occurs by a nucleation-dependent (seeded) polymerization mechanism which is characterized by spontaneous formation of prion amyloid [25, 26]. The spontaneous conversion of the normal cellular isoform of the prion protein, PrP^{C}, into the abnormal isoform, PrP^{Sc}, underlies sporadic forms of prion diseases, including Creutzfeldt-Jakob disease in humans which accounts for approximately 85% of all cases of prion disease [27]. Because familial Cruetzfeldt–Jacob disease or GSS pathology develops spontaneously (i.e., without introduction of an exogenous prion agent), understanding how mutations affect the biophysical properties of prion protein could provide insight into the mechanism of prion protein conversion and the role of the different domains of the molecule involved in the propensity of misfolding and catalysis of the misfolding process. In support of this scenario, we have recently shown that pathogenic truncation mutation (amber), the only known truncated protein that is linked to a prion disease, Gerstmann-Sträussler-Scheinker (GSS) [28, 29] is sufficient to induce in vitro generation of prions using PMCA [17]. The apparent conversion resistance of the full-length prion

protein which contrasts with the behavior of the PrP145Stop variant was explored in this study. The Y145Stop is the only clinically relevant truncation mutation associated with a known prion disease, GSS. Consequently, comparison of the effect of the different polypeptide truncations analyzed here suggest that the N-terminus of PrP^{C} molecule plays a role in seeding and misfolding of mammalian prions. The C-terminal domain obviously cannot mimic all aspects of conversion of the N-terminal region, in which additional cofactors might be required to enhance prion propagation. NMR characterization of the full-length recombinant murine prion protein, moPrP(23-231), showed that the N terminus (residues 23 to ∼120) accounts for most of the PrP flexibly disordered behavior, whereas the C terminus (residues 121–231) encompasses a globular fold [30]. Similar studies of seeded conversion of PrP90-231 could be reproduced using a reduction-oxidation process [31] or under conditions requiring high concentration of denaturants and very vigorous shaking [32]. To date, two types of cofactors - lipids and polyanions have been identified as influencing prion propagation and infectivity, but the precise mechanism remains unclear [33]. The cofactor binding may render the normal PrP^{C} susceptible to conversion or simply facilitate PrP^{Sc}-steered PrP^{C} conversion [34-36]. It can be envisioned that cofactors are required during the initial stages of the conformational transition, whereas these molecules would act by lowering the free energy barrier between PrP^{C} and PrP^{Sc} and triggering conversion [37, 38]. It has been reported that the N-terminus of the protein is important for the interaction with nucleic acids, because mutants lacking different portions of this region presented lower or no affinity for some RNA aptamers [39, 40], and DNA binding was obtained for a rec-PrP(23–144) protein [41]. Taken together these data suggest an essential role of the N-terminus of the

molecule in the mechanism of prion misfolding as well as in protein-protein interactions, whereas the misfolded N-terminal region could be the intermediate that mediates the formation of nascent PrP^{Sc}.

Comparative studies between prion-infected and uninfected cells represent a useful model to understand the cellular and molecular events leading to the formation of PrP^{Sc}. By using the cell lines described here, the ability of Y145Stop to induce prion propagation and infectivity was assessed. Our data demonstrate that Y145Stop subjected to PMCA and used to challenge MDB cells resulted in de novo PrPres, while PrP90-231 and PrP146-231 did not show any PrP conversion efficiency. Moreover, the ability of misfolded Y145Stop to support PrP^{Sc} formation in the cell line is comparable to that of wild type derived from infected brain material. Recent studies showed that bacterially produced N-terminal fragments of Sup35p, when transformed into amyloid fibrils, were able to propagate the prion phenotype to yeast cells [42, 43]. While the infectivity of misfolded Y145Stop in cell culture has not been explored before, several mutant PrPres-like molecules have been generated, some of which have been shown to acquire various biochemical properties of PrPres, but so far, none of them have been shown to be infectious [44, 45]. Moreover, inducing the misfolding of recombinant protein or short PrP synthetic peptides into β sheet-rich structures exhibiting some of the biochemical and biological properties of PrPres [31, 46, 47], but very small percentage of the protein altered in vitro adopted the infectious folding. In contrast to these results our study demonstrated that misfolded recombinant Y145Stop is sufficient to support PrP^{Sc} amplification, although the cells are more susceptible to MDB^{CWD} cell lysates is not

clear. It could be possible explained that some strains can replicate in one particular cell line [48] and only some cells from a culture become infected and subcloning could improve the susceptibility to prions. Importantly, there was no detectable infectivity associated in inoculated cells with De146-231 or De90-231, excluding any significant effect of residual inoculum. Altogether these findings led to conclude that a truly infectious, PrP^{CWD} was propagated from Y145Stop that seeded PrP^{C} in MDB cells.

MATERIALS AND METHODS

Expression and Purification of the recombinant PrP145Stop and C-terminal fragments of PrP

Expression vectors for recombinant De- and Sh-Y145Stop were prepared as described previously [17]. Segments for the expression of c-terminals were amplified using the forward primer as 5'- AAACCATGGGGTCAAGGTGGTACCCACAGT -3' for De-90-231 and 5'- AAACCATGGGAGGACCGTTACTATCGTGAA -3' for De-146-231, while 5'- CCCAAGCTTTCAACTTGCCCCTCTTTGGTAATA -3' as the reverse primer for both terminals. Open reading frames were subsequently cloned into NcoI and HindIII (NEB) restriction sites of the pET-41 a vector (Novagen). All proteins were expressed in *E. coli* and purified by cobalt ion affinity chromatography essentially as previously described [17]. Unlike the Y145Stop purification method based on the affinity of the octapeptide repeats for transition-metal cations, the C-terminal fragments were expressed as His-tagged proteins. The N-terminal polyhistidine tag was cleaved using enterokinase (Novagen/EMD Biosciences, Madison, WI). The free polyhistidine tag was then removed by dialysis against ultrapure water and the samples were buffer exchanged using Zeba-desalting columns (Pierce, Rockford, IL, USA), further concentrated to 0.5 mg/ml and fresh frozen at -80^{o}C.

Preparation of purified normal and pathological isoforms of prion protein from brain tissues

PrP^{C} and PrP^{CWD} from deer brains were purified by sucrose density-gradient centrifugation and subjected to ultracentrifugation method as described[17, 49]. In brief,

brain tissue was sliced and rinsed with cold PBS to remove the residual blood. Two syringes connected with a Three-Way Stopcock with Male Luer Slip Adapter (Baxter Healthcare Corp, Deerfield, IL, USA) were used to prepare 10% w/v brain suspension using cold 10% sarcosine (TEND) that is supplemented with 1mM DTT and ethylenediaminetetraacetic acid (EDTA)-free Protease Inhibitors cocktail (Roche, Mannheim, Germany) at each step where it was needed. Aliquots of this suspension were homogenized then transferred on ice for one hour. The mixture was subjected to centrifugation at 3,000 ×*g* for 10 minutes to pellet of cellular debris. The supernatants were transferred to new Optiseal tubes (Beckman Coulter, Fullerton, CA) and centrifuged through 1 M sucrose cushion for 2 h 30 min at 4^{o}C at 100 000 ×*g*. This pellet consists of a microsomal/ synaptosomal membrane fraction containing PrP^{C}. Then the pellets were rinsed using 10% NaCl, 1% SB 3-14 (TEND+ PIs). DNAase I and RNAase treatment were used to remove nucleic acids from the aliquots. At this point, a portion of the material was digested with different concentrations (1, 5, 10 µg/ml) of proteinase K enzyme. The suspension was then subjected to centrifugation at 125,755 ×*g* for 2 hours and pellets were rinsed using 0.5% SB 3-14/1X PBS and finally re-suspended in 1 ml 2% sarkosyl. The protein concentration was adjusted using bicinchonic acid assay (Pierce, Rockford, IL, USA) to 0.5-1 mg/ml.

Preparation of In vitro converted prions using PMCA

The procedures for in vitro conversion of the different samples by PMCA have been described [17], and used 0.2 ml thin wall PCR tube strips as 80-µl solutions containing PBS with 0.05% (wt/vol) SDS and 0.05% Triton X-100. PK-resistant fragments seeded

by PrP^{Sc} were generated with seed-to-substrate ratios of 1:100 (40 ng of PrP^{Sc}). The tube strips were placed in floating rack in the Sonicator (Misonix Model 4000, Farmingdale, New York) cup horn with water at 37°C and were subjected to repeated cycles of sonication. The samples were subjected to two rounds of PMCA reaction, each of them consisting of 24 cycles with 40 sec intermittent sonication at 100% power (16 min total sonication time), 59 min 20 sec incubations between each sonication. After the first round, an aliquot of the amplified samples was taken and diluted 10-fold into the conversion buffer containing the monomeric protein (0.5 mg/ml) as substrate. A second round of 24 cycles of sonication and incubation as described above was repeated. Buffer, vector control and unseeded sonication were used to test the tendency of the samples for spontaneous misfolding and/or contamination.

Cell Culture

MDB and MDB^{CWD} cells were a gift from Dr. Byron Caughey (Rocky Mountain Laboratories, Hamilton) and expanded for 2 to 3 passages before being frozen. Cells were grown and maintained using standard protocols as described [24]. Briefly, cells were cloned by dilution into 25-cm^2 flasks and grown in Opti-MEM reduced serum medium (Invitrogen, CA, USA) containing L-glutamine and supplemented with 10% fetal bovine serum, 1x glutamine, and penicillin and streptomycin. Cells were scraped using a cellstripper (cellgro Mediatech, Inc., Herndon, VA) and passaged at a 1:10 dilution from a confluent plate, thereafter grown for 4 days in Opti-MEM.

Inoculation of cells

Cultures were split 1:10 into 24-well tissue culture plates. Generally, either a purified brain PrP from normal and infected brain or PMCA-subjected recombinant proteins of Y145Stop and full length PrP^C were added to the medium at a 1:30 ratio. Approximately 900 µl of the inoculated medium was added to each well. Cells were grown in the presence of the inoculum for 4 days before splitting at 1:10 ratio. Afterward, cells were grown in uninoculated medium. In cases where cell growth was slow, cultures were split at lower ratios. Plastic coverslips were obtained from Nunc, Inc. (Rochester, NY).

Cell blotting

Cell blot analyses were performed using standard procedures, as described [50]. Cells were blotted onto a nitrocellulose (NC) membrane from coverslips by applying firm pressure for 30 s. The NC membrane was air-dried and incubated in a lysis buffer (0.5% deoxycholate, 0.5% Triton X-100, 150 m*M* NaCl, and 10 m*M* Tris-HCl, pH 7.5) with or without 3 µg/ml PK for 1.5 hrs at 37°C. The NC membrane was washed in distilled water and incubated for 20 mins with 0.1M Pefabloc (Sigma) at room temperature. The membrane was immersed in denaturing buffer (3 *M* guanidine isothiocyanate and 10 m*M* Tris-HCl, pH 8.0) for 10 mins, washed three times in water, and blocked in 5% nonfat dry milk in PBST for 2 hrs. After blocking, the membrane was incubated with the mAbs 1E4 (1:1000; Fitzgerald Industries International, Concord, MA, USA) or F89 (1:2000; Abcam Inc, Cambridge, MA, USA) overnight in 5% nonfat dry milk and PBS with 0.1% Tween 20. All blots were developed using a chemiluminiscent substrate (Supersignal West Pico Chemiluminescent Substrate; Pierce) for 5 to 15 mins.

Western blotting of cell lysates

Confluent 25 cm^2 flasks were rinsed 3 times with 3-5 ml of ice cold Dulbecco's Phosphate-Buffered saline with calcium and magnesium (D-PBS) and then lysed by the addition of 1 ml of cold lysing buffer. The nuclear pellet was removed, and the protein concentration was measured using BCA assay (Pierce). Samples of equal protein amount and volumes were digested with 20 µg/ml PK at a ratio of 1:100 (2 µg of PK for 200 µg of protein) at 37°C for 1 h. Digestion was stopped with 1 mM Pefabloc, and samples were pelleted by centrifugation at 20,000 ×*g* at 4°C for 30 min. Digestion was stopped with 1 mM Pefabloc, and samples were centrifuged at 20,000 ×*g* at 4°C for 30 min. Pellets were dissolved in 20 µl of lysis buffer and 20 µl of 2× loading buffer and then boiled for 10 min before loading on 4-20% SDS-PAGE precast gels (Bio-Rad Laboratories Inc, Hercules, CA, USA). Western blots were performed according to standard procedures. Blots were probed with mAbs 1E4 (Fitzgerald Industries International, Concord, MA, USA) and detected with the ECL system.

Figures

Figure 7: Immunoblots of recombinant C-terminals (De-90-231 and De 146-231) after PMCA. Lane 1 and 4 rec De-90-231 and De 146-231, respectively, submitted to two successive rounds of PMCA without PK digestion. In lane 2 and 5, samples were digested with PK and did not show any detectable spontaneous conversion. Seeding purified PrP^{C} from brain tissue of deer with PMCA treated De-90-231; lane 3, or De-146-231; lane 6, showed no detectable PrPres after PK digestion. Positions of the molecular mass markers are designated in kDa.

Figure 7: Immunoblots of recombinant C-terminals (De-90-231 and De 146-231) after PMCA

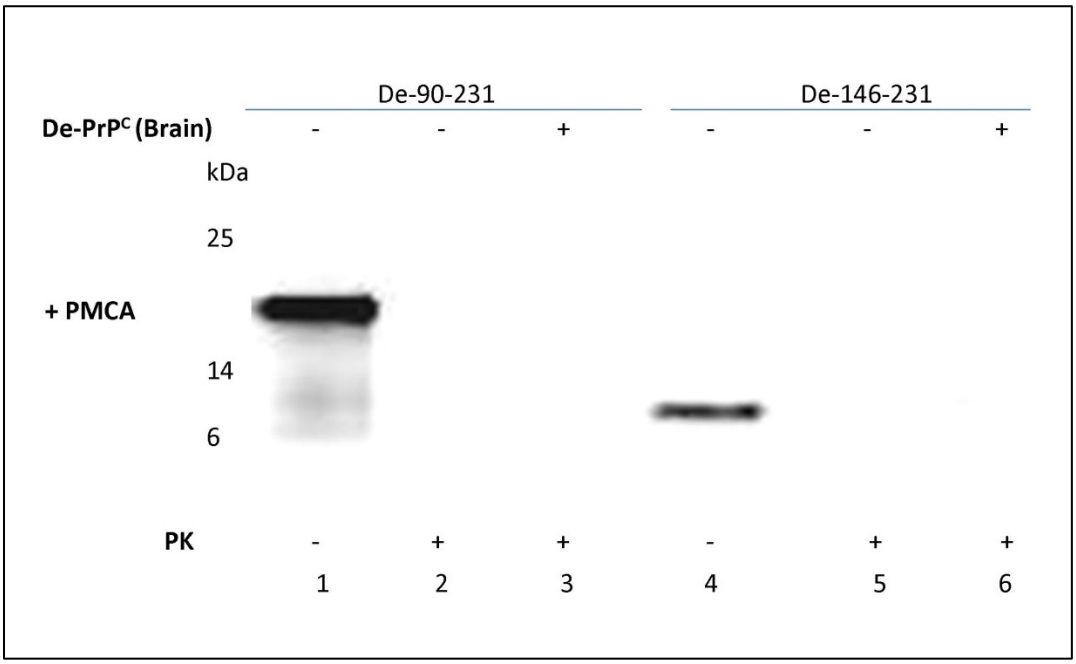


Figure 8: Flow chart depicting the different inocula used in the study. The MDB cell line expressing PrP^{C} was prepared in 24-well plates. Cells were grown in the presence of an inoculum of PMCA-misfolded Y145Stop for 4 days before being split. Cells inoculated with Purified PrP^{C} from brain and PMCA assay buffer were used as a negative control. As a positive control, MDB^{CWD} cell lysates or purified PrP^{CWD} from brain tissue were used for inoculation.

Figure 8: Flow chart depicting the different inocula used in the study.

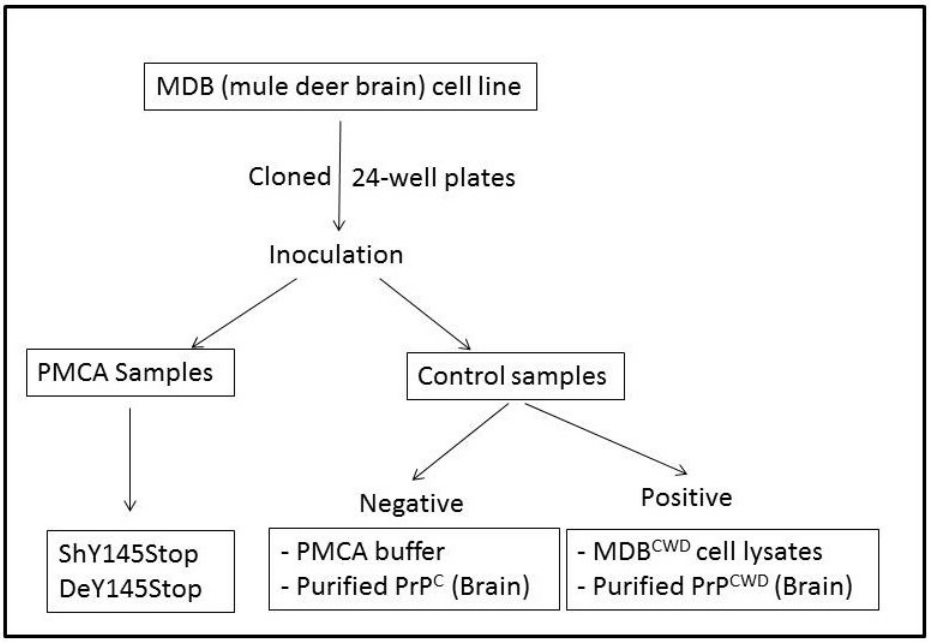


Figure 9: MDB cells with the different inocula. Sublines were passaged for 24 days. (A) Each circular blot in the figure is from a single and independently inoculated and passaged cell population, with paired blots from same subline shown side by side. Cloned MDB^{CWD} showed persistent PrP^{CWD} production. Susceptible MDB cells produced detectable PrP^{Sc} when inoculated with partially purified PrP^{CWD} and cell lysates of the MDB^{CWD} cells. The negative control inocula, PMCA buffer and purified De-PrP^{C} from brain tissue, showed no detectable PrP^{Sc} in the infected MDB cells. (B) Prion conversion in MDB cells by treatment with misfolded De-Y145Stop by PMCA. After inoculation with Y145Stop, the MDB cells were further subcloned, and analyzed. Each circular blot represents a separate subline derived from inoculated clone performed in duplicate; 6 sublines produced detectable amount of proteinase K-resistant PrP. (C) Western blots performed on cell lysates from clones showed in Panel B. The product of proteinase K-treated lysates, 200 μg (approximately 5×10^6 cells) of total protein was loaded in each lane. All positive clones with cell blots showed presence of PrP^{res} in the cell lysate.

Figure 9: MDB cells with the different inocula

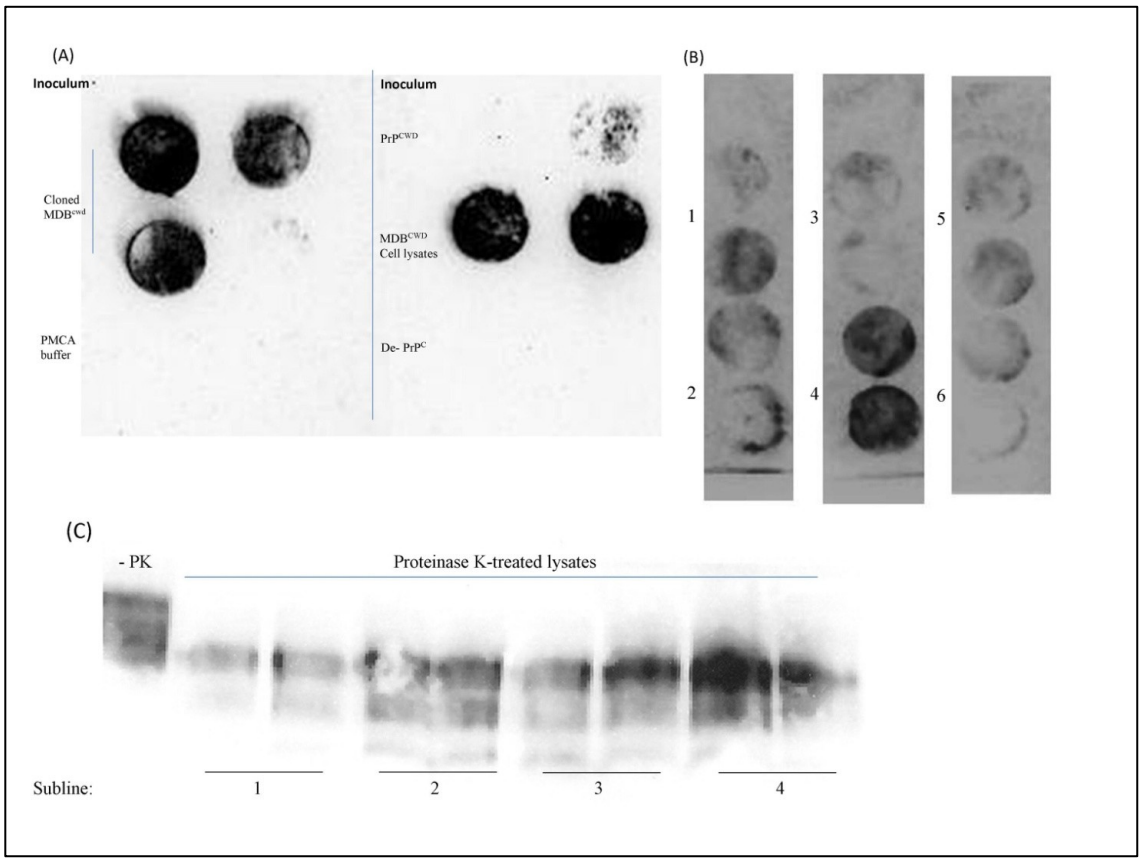


Figure 10: Time course analysis for accumulation of PrPres in Y145Stop seeded MDB cells. A susceptible MDB subline was inoculated with misfolded Y145Stop (Left column) or partially purified PrP^{CWD} (right column). At 3-4 days intervals after inoculation, cells were passaged and aliquots were plated for cell blotting. After 2 passages (8 days), proteinase K-resistant was barely detectable. The signal increased thereafter indicating *de novo* formation of PrP^{Sc}. Same pattern of PrP^{Sc} accumulation was noticed with culture inoculated with PrP^{CWD}.

Figure 10: Time course analysis for accumulation of PrPres in Y145Stop seeded MDB cells

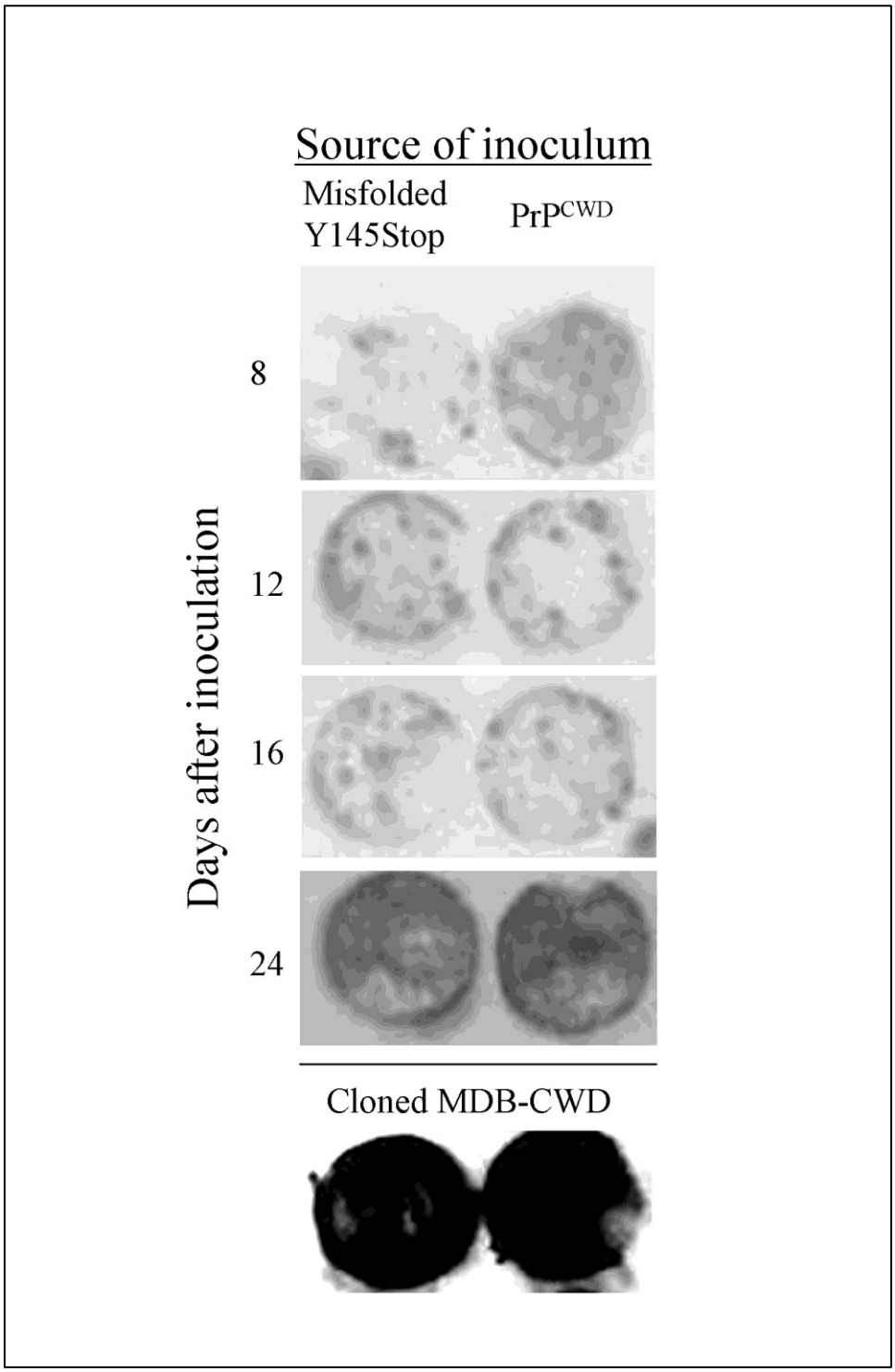
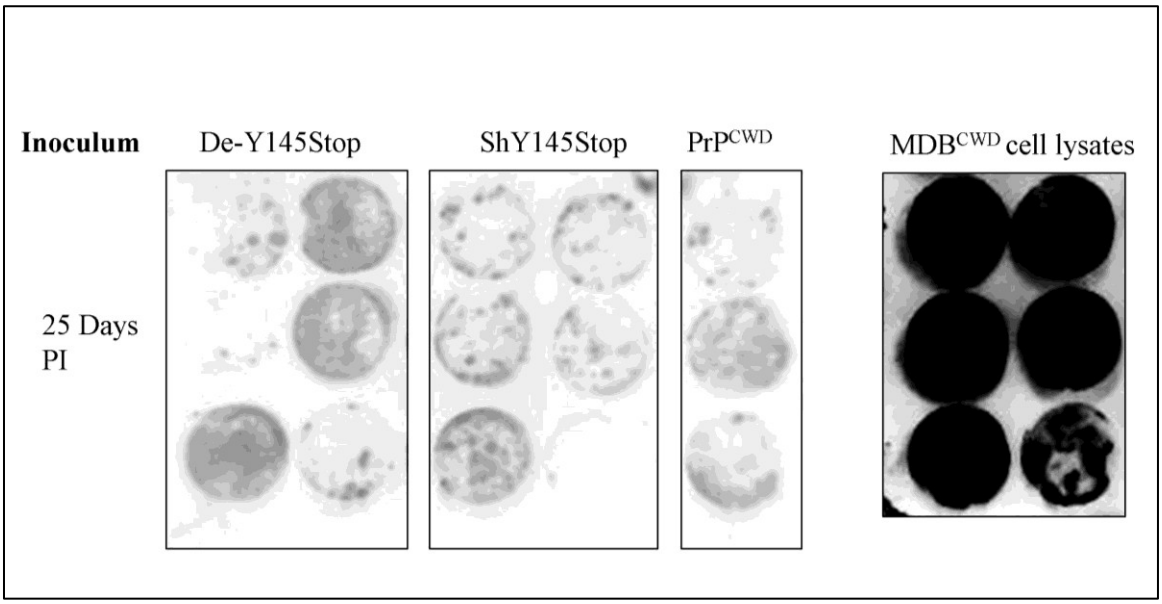


Figure 11: Comparisons of misfolding induced by Y145Stop from deer or sheep in MDB cells. MDB susceptible cells were inoculated with misfolded DeY145Stop (deer), ShY145Stop (sheep), and partially purified PrP^{CWD} and compared to cells inoculated with MDB^{CWD} cell lysates. After 6 passages, sublines inoculated with misfolded DeY145Stop and ShY145Stop retain PrPres production. The cellblots of inoculated cells with DeY145Stop and ShY145Stop showed as much PrP^{Sc} as PrP^{CWD} inocula. MDB cells were more susceptible to MDB^{CWD} cell lysates than the misfolded Y145Stop inocula.

Figure 11: Comparisons of misfolding induced by Y145Stop from deer or sheep in MDB cells



References

1. Prusiner, S.B., *Prions.* Proc Natl Acad Sci U S A, 1998. **95**(23): p. 13363-83.
2. Pan, K.M., et al., *Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins.* Proc Natl Acad Sci U S A, 1993. **90**(23): p. 10962-6.
3. Morillas, M., D.L. Vanik, and W.K. Surewicz, *On the mechanism of alpha-helix to beta-sheet transition in the recombinant prion protein.* Biochemistry, 2001. **40**(23): p. 6982-7.
4. Prusiner, S.B., *Molecular biology of prion diseases.* Science, 1991. **252**(5012): p. 1515-22.
5. Meier, P., et al., *Soluble dimeric prion protein binds PrP(Sc) in vivo and antagonizes prion disease.* Cell, 2003. **113**(1): p. 49-60.
6. Prusiner, S.B., et al., *Transgenetic studies implicate interactions between homologous PrP isoforms in scrapie prion replication.* Cell, 1990. **63**(4): p. 673-86.
7. Scott, M., et al., *Propagation of prions with artificial properties in transgenic mice expressing chimeric PrP genes.* Cell, 1993. **73**(5): p. 979-88.
8. Saa, P., J. Castilla, and C. Soto, *Ultra-efficient replication of infectious prions by automated protein misfolding cyclic amplification.* J Biol Chem, 2006. **281**(46): p. 35245-52.
9. Saborio, G.P., B. Permanne, and C. Soto, *Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding.* Nature, 2001. **411**(6839): p. 810-3.
10. Wang, F., et al., *Generating a prion with bacterially expressed recombinant prion protein.* Science, 2010. **327**(5969): p. 1132-5.
11. Deleault, N.R., et al., *Formation of native prions from minimal components in vitro.* Proc Natl Acad Sci U S A, 2007. **104**(23): p. 9741-6.
12. Collins, S., C.A. McLean, and C.L. Masters, *Gerstmann-Straussler-Scheinker syndrome,fatal familial insomnia, and kuru: a review of these less common human transmissible spongiform encephalopathies.* J Clin Neurosci, 2001. **8**(5): p. 387-97.
13. Ghetti, B., et al., *Gerstmann-Straussler-Scheinker disease and the Indiana kindred.* Brain Pathol, 1995. **5**(1): p. 61-75.
14. Goldfarb, L.G. and P. Brown, *The transmissible spongiform encephalopathies.* Annu Rev Med, 1995. **46**: p. 57-65.
15. Jodoin, J., et al., *Loss of anti-Bax function in Gerstmann-Straussler-Scheinker syndrome-associated prion protein mutants.* PLoS One, 2009. **4**(8): p. e6647.
16. Kitamoto, T. and J. Tateishi, *Human prion disease and human prion protein disease.* Curr Top Microbiol Immunol, 1996. **207**: p. 27-34.
17. Abdallah, A., et al., *Y145Stop is sufficient to induce de novo generation prions using protein misfolding cyclic amplification.* Prion, 2012. **6**(1).
18. Clarke, M.C. and D.A. Haig, *Evidence for the multiplication of scrapie agent in cell culture.* Nature, 1970. **225**(5227): p. 100-1.
19. Butler, D.A., et al., *Scrapie-infected murine neuroblastoma cells produce protease-resistant prion proteins.* J Virol, 1988. **62**(5): p. 1558-64.

20. Race, R.E., L.H. Fadness, and B. Chesebro, *Characterization of scrapie infection in mouse neuroblastoma cells.* J Gen Virol, 1987. **68 (Pt 5)**: p. 1391-9.
21. Schatzl, H.M., et al., *A hypothalamic neuronal cell line persistently infected with scrapie prions exhibits apoptosis.* J Virol, 1997. **71**(11): p. 8821-31.
22. Sabuncu, E., et al., *PrP polymorphisms tightly control sheep prion replication in cultured cells.* J Virol, 2003. **77**(4): p. 2696-700.
23. Vorberg, I., et al., *Susceptibility of common fibroblast cell lines to transmissible spongiform encephalopathy agents.* J Infect Dis, 2004. **189**(3): p. 431-9.
24. Raymond, G.J., et al., *Inhibition of protease-resistant prion protein formation in a transformed deer cell line infected with chronic wasting disease.* J Virol, 2006. **80**(2): p. 596-604.
25. Come, J.H., P.E. Fraser, and P.T. Lansbury, Jr., *A kinetic model for amyloid formation in the prion diseases: importance of seeding.* Proc Natl Acad Sci U S A, 1993. **90**(13): p. 5959-63.
26. Caughey, B., *Interactions between prion protein isoforms: the kiss of death?* Trends Biochem Sci, 2001. **26**(4): p. 235-42.
27. Hill, A.F., et al., *Molecular classification of sporadic Creutzfeldt-Jakob disease.* Brain, 2003. **126**(Pt 6): p. 1333-46.
28. Kitamoto, T., R. Iizuka, and J. Tateishi, *An amber mutation of prion protein in Gerstmann-Straussler syndrome with mutant PrP plaques.* Biochem Biophys Res Commun, 1993. **192**(2): p. 525-31.
29. Ghetti, B., et al., *Vascular variant of prion protein cerebral amyloidosis with tau-positive neurofibrillary tangles: the phenotype of the stop codon 145 mutation in PRNP.* Proc Natl Acad Sci U S A, 1996. **93**(2): p. 744-8.
30. Riek, R., et al., *NMR characterization of the full-length recombinant murine prion protein, mPrP(23-231).* FEBS Lett, 1997. **413**(2): p. 282-8.
31. Lee, S. and D. Eisenberg, *Seeded conversion of recombinant prion protein to a disulfide-bonded oligomer by a reduction-oxidation process.* Nat Struct Biol, 2003. **10**(9): p. 725-30.
32. Baskakov, I.V., *Autocatalytic conversion of recombinant prion proteins displays a species barrier.* J Biol Chem, 2004. **279**(9): p. 7671-7.
33. Kubota, T., et al., *Direct evidence of generation and accumulation of beta-sheet-rich prion protein in ScN2a cells de novo illuminated by human IgG1 antibody recognizing beta-form but not alpha-form of prion protein.* J Biol Chem, 2012.
34. Wong, C., et al., *Sulfated glycans and elevated temperature stimulate PrP(Sc)-dependent cell-free formation of protease-resistant prion protein.* EMBO J, 2001. **20**(3): p. 377-86.
35. Gomes, M.P., et al., *Prion protein complexed to N2a cellular RNAs through its N-terminal domain forms aggregates and is toxic to murine neuroblastoma cells.* J Biol Chem, 2008. **283**(28): p. 19616-25.
36. Wang, F., et al., *Lipid interaction converts prion protein to a PrPSc-like proteinase K-resistant conformation under physiological conditions.* Biochemistry, 2007. **46**(23): p. 7045-53.
37. Baskakov, I.V., et al., *Folding of prion protein to its native alpha-helical conformation is under kinetic control.* J Biol Chem, 2001. **276**(23): p. 19687-90.

38. Cordeiro, Y. and J.L. Silva, *The hypothesis of the catalytic action of nucleic acid on the conversion of prion protein.* Protein Pept Lett, 2005. **12**(3): p. 251-5.
39. Weiss, S., et al., *RNA aptamers specifically interact with the prion protein PrP.* J Virol, 1997. **71**(11): p. 8790-7.
40. Sekiya, S., et al., *Characterization and application of a novel RNA aptamer against the mouse prion protein.* J Biochem, 2006. **139**(3): p. 383-90.
41. Gabus, C., et al., *The prion protein has DNA strand transfer properties similar to retroviral nucleocapsid protein.* J Mol Biol, 2001. **307**(4): p. 1011-21.
42. King, C.Y. and R. Diaz-Avalos, *Protein-only transmission of three yeast prion strains.* Nature, 2004. **428**(6980): p. 319-23.
43. Tanaka, M., et al., *Conformational variations in an infectious protein determine prion strain differences.* Nature, 2004. **428**(6980): p. 323-8.
44. Lehmann, S. and D.A. Harris, *Two mutant prion proteins expressed in cultured cells acquire biochemical properties reminiscent of the scrapie isoform.* Proc Natl Acad Sci U S A, 1996. **93**(11): p. 5610-4.
45. Chiesa, R., et al., *Neurological illness in transgenic mice expressing a prion protein with an insertional mutation.* Neuron, 1998. **21**(6): p. 1339-51.
46. Jackson, G.S., et al., *Reversible conversion of monomeric human prion protein between native and fibrilogenic conformations.* Science, 1999. **283**(5409): p. 1935-7.
47. Baskakov, I.V., et al., *Self-assembly of recombinant prion protein of 106 residues.* Biochemistry, 2000. **39**(10): p. 2792-804.
48. Mahal, S.P., et al., *Prion strain discrimination in cell culture: the cell panel assay.* Proc Natl Acad Sci U S A, 2007. **104**(52): p. 20908-13.
49. Wang, P., et al., *Selection and characterization of DNA aptamers against PrP(Sc).* Exp Biol Med (Maywood), 2011. **236**(4): p. 466-76.
50. Bosque, P.J. and S.B. Prusiner, *Cultured cell sublines highly susceptible to prion infection.* J Virol, 2000. **74**(9): p. 4377-86.

CHAPTER 4: Y145Stop propagation in cell culture is associated with apoptotic signaling pathway.

All prion diseases share a common molecular mechanistic feature in that PrP^{C} undergoes conformational conversion to the disease associated PrP^{Sc} in host cells during pathogenesis. Some studies suggest that PrP^{Sc} can cause pathologic effects to the neuronal cells by triggering signal transduction cascades for apoptosis. Since it has been shown that Y145Stop can induce PrP^{Sc} accumulation in cell culture, we hypothesized that Y145Stop-induced cell death is associated with apoptotic signal pathway. We investigated the intracellular signaling responsible for Y145Stop-dependant cell death. The toxic effects of misfolded Y145Stop and recombinant protein full-length PrP^{C} was compared to the disease-associated prion (PrP^{Sc}) extracted from the brain tissue of deer on a mule deer brain (MDB) cell line. In these cells, only misfolded recombinant Y145Stop treatment induced apoptotic cell death and activation of caspases-3. Furthermore, the involvement of mitogen-activated protein (MAP) kinase in this phenomenon was examined. Exposure of MDB cells to misfolded Y145Stop caused p38 activation that was blocked by a p38MAPK inhibitor and led to the inhibition of apoptotic cell death. In conclusion, these data indicate that in addition to induction of infectivity in cell culture, exogenously added misfolded Y145Stop triggers DNA cleavage and cell death via the activation of both capsase-3 and p38 MAP kinase. The Y145Stop recapitulates cell signaling events similar to those induced by disease associated prions further establishing a biological role for the misfolded Y145Stop.

INTRODUCTION

Prions are thought to propagate by an autocatalytic process in which PrP^{Sc} self-replicates by serving as a template to the conformational rearrangement of endogenous PrP^{C}. But the molecular events through which prion infection and the resulting accumulation of PrP^{Sc} lead to prion pathology remain unclear [1, 2]. Although pathogenesis is associated with PrP^{Sc} accumulation, it is not clear whether the gain of toxic PrP^{Sc} function is responsible for the pathologic phenotypes such as neurotoxicity, cell death, and neurodegeneration. Some studies suggest that PrP^{Sc} can cause pathologic effects by triggering signal transduction cascades for apoptosis [3, 4]. Alternatively, the loss of physiological PrP^{C} function may be associated with an increased neuronal vulnerability. There is experimental evidence suggesting that PrP^{C} may have a neuroprotective capacity, which is lost upon the formation of misfolded conformers [5-11]. A cytoprotective role of PrP^{C} against internal or environmental stresses that initiate apoptosis has been proposed. This anti-apoptotic potential is based on the capacity of PrP^{C} to inhibit the action of pro-apoptotic protein Bax that affects the release of cytochrome c [12]. Therefore, neuronal apoptosis is one of the main characteristics of prion diseases whether PrP^{Sc} accumulation causes a loss or gain of function of the prion protein.

To study the molecular events crucial for the conversion of PrP^{C} to PrP^{Sc} and neurotoxicity, many studies have been performed using either synthetic peptides or recombinant PrP molecule expressed in heterologous systems. From the analysis of the biological activity of different amino acidic segments, a peptide corresponding to the residues 106-126 of the PrP sequence (PrP 106-126) has been largely used to explore the

neurotoxic mechanisms underlying the prion diseases [13-15]. PrP 106-126 has been shown to be highly hydrophobic and fibrillogenic *in vitro*, and is partially resistant to proteolysis [13, 16, 17]. Moreover, this peptide was reported to induce apoptotic neuronal death [13, 18-21]. Thus, it was proposed that this peptide contains the death motif within the PrP sequence. However, no clinically-relevant prion disease is associated with this neurotoxic fragment. Furthermore, Fioriti et al. have shown that PrP 106-126 did not induce detergent-insoluble and protease-resistant PrP, not did it alter its membrane topology or cellular distribution [22]. Therefore, the relevance of PrP 106-126 as a model for studying prion-induced effects has been subjected to debate. Interestingly, the PrP106-126 fragment is a part of a clinically-relevant truncation mutation, PrPY145Stop, which is associated with a known prion disease; Gerstmann-Sträussler-Scheinker (GSS) [23, 24]. We generated a recombinant protein encompassing residues 23-144 of deer PrP (DeY145Stop) that, in its native form, is a soluble monomer. Utilizing protein misfolding cyclic amplification (PMCA) which converts Y145Stop in PK-resistant PrP^{Sc} renders the peptide insoluble and partially resistant to proteolysis [25]. These features allow Y145Stop to acquire biological activities *in vitro*, inducing conversion of purified mammalian PrP^{C} into protease resistant isoforms [25] and triggering the infectivity in cell culture (unpublished data), and thus, represents a valuable model to study misfolding of mammalian prion and its toxic activity.

In this study the toxic effect of DeY145Stop in a brain-derived cell line (MDB) [26] and the involvement of caspases and MAP kinases in this phenomenon were investigated. Here, Y145Stop induced apoptosis in MDB cells through the activation of caspases 3 and p38 MAP kinase has been reported. These data have significant

implications on the role of the N-terminus of PrP^C molecule in seeding and misfolding of mammalian prions.

RESULTS

PrPY145Stop induces cell death in MDB cells

PrPY145Stop was observed to induce cell death in a dose-dependent manner over time as measured by the MTT assay (Figure 12 a-d). The tetrazolium compound MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) is converted to a formazan product by mitochondrial enzymes, which become inactive as the cell dies. Measurement of this formazan product is an indicator of cell viability. Using the MTT test, the time and dose dependency of PrPY145Stop toxicity, recombinant protein full-length PrP^C and N-terminal truncated form of prion protein; PrP146-231 compared to PrP^{CWD} were quantified. Cells inoculated with Purified PrP^C from brain and PMCA assay buffer were used as negative controls. MDB cell viability was significantly affected after 3 days of exposure to PrPY145Stop (50 μM) (Figure 12 a) and was reduced to less than 55% of the control value after treatment with 400 μM PrPY145Stop (Figure 12 c). A similar pattern of cell death was detected in PrP^{CWD} treated cells. Conversely, no signs of cell death were detectable in PrP146-231, Purified PrP^C from brain, rec-PrP^C, or PMCA assay buffer treated cells.

The mechanism of cell death induced by PrPY145Stop is by apoptosis

A specific cell death detection ELISA was used to illustrate cell death and differentiate between apoptotic and necrotic cells. By measuring the amount of mono- and

oligonucleosomes released in the cytosol by apoptotic endonucleases, PrPY145Stop and PrP^{CWD} (200 μM) caused a time dependent and significantly detectable DNA cleavage after 2 days of treatment and reaching the highest value after 4 days (approximately 275% for Y145Stop and 300% PrP^{CWD} over control) (Figure 13). No effects were observed upon treatment with rec-PrP^{C} and PMCA assay buffer.

Caspase-3 involvement in the toxic effects in response to PrPY145Stop

Because PrPY145Stop-induced cell death showed some features of apoptosis, the activation of Caspase-3 in the toxic effect of the peptide was analyzed. We assessed Caspase-3 activation by measuring the cleavage of the fluorigenic substrate; acetyl Asp-Glu-Val-Asp 7-amido-4-methylcoumarin (Ac-DEVD-AMC). The proteolytic activity of Caspase-3 was increased significantly after 2 hours of treatment with 200 μM PrPY145Stop and increased over time (Figure 14 a). Also, we measured the effects of PrPY145Stop on MDB cells DNA cleavage in the presence of Z-VAD-FMK, a general caspase blocker, and Z-DEVD-FMK a more selective caspase-3 inhibitor. The coincubation with the two caspase blockers inhibited the DNA fragmentation caused by 3 days of exposure to PrPY145Stop (Figure 14 b).

Role of p38MAP kinases in the proapoptotic effects of PrPY145Stop

Activation of p38 MAP kinase is one of the early intracellular events that causes mitochondrial depolarization and the apoptotic cascade [27]. Therefore, the hypothesis that p38 MAP kinase is involved in Y145Stop cell death was tested. The activation of p38 MAP kinase by Y145Stop was directly assessed by western blot. We compared the

ratio of the phosphorylated enzyme to the total p38 levels using the densitometric analysis. Treatment for 3 days of Y145Stop (100 μM) caused a significant activation of p38 (Figure 15 a). Further, we used p38 MAP kinase blocker to investigate the involvement of MAP kinase p38 in the MDB apoptotic death induced by Y145Stop. Y145Stop and p38 blocker were added together and apoptosis was assessed by ELISA after 3 days. Adding the p38 blocker was able to prevent the apoptotic process (Figure 15 b). These data confirm that the activation of p38 MAP kinase pathway is a critical step for the execution of apoptosis in our cell model.

DISCUSSION

Y145Stop is the only truncated prion molecule that is linked to an autosomally dominant inherited genetic TSE, the causing mutation for the Gerstmann-Sträussler-Scheinker syndrome [23, 24]. Thus, a recombinant prion protein variant consisting of amino acid residues 23-144, PrPY145Stop, represents the N-terminus of the prion protein. Using PrPY145Stop, it has been shown that fundamental aspects of mammalian prion propagation, including the spontaneous formation of prion amyloid fibrils and seeding capability, can be reproduced in vitro in a manner similar to PrP^{Sc} [25]. The toxicity of the PrPY145Stop peptide in cell culture has not been demonstrated previously. Therefore, the aim of the work is to investigate the molecular aspects of the cell death machinery triggered by PrPY145Stop using the MDB cell line.

The physical nature of the recombinant proteins is an important factor in its toxicity. It has been demonstrated that the toxicity of PrP106-126 is related to its ability to form aggregates [13, 28]. Even though some other fibrillar peptides such as PrP106-

114 and PrP127-147 were not toxic, indicating that fibrillogenic properties alone are not sufficient for toxicity [29]. Recently it have been shown that spontaneous aggregation of PMCA misfolded PrPY145Stop using the Thioflavin T (ThT) assay and found that the peptide aggregated after 8 hours and increased its level of aggregation with time [25]. In addition to the fibrillogenic properties of Y145Stop, the present study demonstrates that exposure of MDB cells to Y145Stop significantly impaired the cell viability in a time- and concentration dependent manner through the induction of apoptosis. Apoptotic cell death was detectable already after two days of treatment with 200 μM PrPY145Stop or PrP^{CWD} as observed by an ELISA test that detects the presence of cytosolic oligonucleosome-sized DNA with high specificity and sensitivity [30]. On the other hand, rec-DePrP and PrP146-231 did not show any sign of cell toxicity. Since the cell death triggered by PrPY145Stop showed hallmarks of apoptosis, we studied the involvement of the caspases in the MDB cell death induced by PrPY145Stop in comparison to PrP^{CWD} extracted from infected brain tissue. To address this issue, whether PrPY145Stop treatment caused a direct activation of caspase-3 and if the inhibition of these enzymes could prevent the cell death induced by the peptide was investigated. The data shown here indicate that PrPY145Stop significantly increased caspase-3 activity. The caspase blocker Z-VAD-FMK and the selective caspase-3 inhibitor Z-DEVD-FMK blocked the DNA breakdown induced by 3 days of exposure to PrPY145Stop. The evidence from the above-mentioned reports support the data about the correlation between caspase activation, apoptosis, and cell death we have obtained in our cell model.

Beside caspases, a role for the mitogen-activated protein kinases (MAP kinases) in the control of several degenerative processes is now emerging [31]. MAP kinases are

serine/threonine protein kinases that rule the signaling of many extracellular stimuli from the plasma membrane to the nucleus; these enzymes belong to a superfamily that follows the extracellular signal-regulated kinases (ERK1/2), the Jun N-terminal kinases (JNKs), and the p38 MAP kinases [32]. It has been shown that p38-MAPK signaling promotes cell death [33, 34], whereas it has also been shown that p38-MAPK cascades enhance survival [35], and cell growth [36]. Western blot analysis showed an increase of the immunoreactivity for the phosphorylated/activated p38 MAP kinase when MDB cells were exposed to PrPY145Stop. Moreover, the co-treatment of the MDB cells with PrPY145Stop in the presence of the p38 inhibitors SB203580 fully blocked the apoptosis induced by three days of treatment with PrPY145Stop. These results supported the hypothesis that p38 MAP kinase is involved in MDB cell death and indicates that the increase of p38 phosphorylation plays a critical role in the cell death triggered by the peptide.

In summary, the data presented here indicate that fibrillogenic aggregation of misfolded PrPY145Stop is associated with apoptotic cell death. The toxic activity of the peptide is dependent, in part, on the activation of caspase and the p38 MAP kinase pathway.

MATERIALS AND METHODS

Recombinant PrPY145Stop and PrP146-231

The recombinant PrP proteins were obtained and purified as previously described [25]. Before addition to cultures, the recombinant proteins were subjected to two rounds of PMCA [25].

Cell culture

MDB cells were cultured in Opti-MEM reduced serum medium (Invitrogen, CA, USA) containing L-glutamine and supplemented with 10% fetal bovine serum, 1x glutamine, and penicillin and streptomycin; the cells were maintained at 37^{o}C in a humidified incubator under 95% air and 5% CO_2. All treatments were performed by a single administration, directly into the wells from the stock solutions to reach the desired concentration. When being harvested for analysis cells were lifted using cellstripper (Cellgro Mediatech, Inc., Herndon, VA).

Preparation of purified normal and pathological isoforms of prion protein from brain tissues

PrP^{C} and PrP^{CWD} from deer brains were purified by sucrose density-gradient centrifugation and subjected to ultracentrifugation method as described [25, 37].

Chemical Reagents

The caspase-3 substrate Ac-DEVD-AMC, the caspase blockers (Z-VAD-FMK and Z-DEVD-FMK) were purchased from Calbiochem (EMD Millipore, Billerica, MA). SB203580 (p38 Inhibitor) was obtained from Cell Signaling Technology (Danvers, MA, USA).

MTT Assay

Cytotoxicity was assessed by the conversion of MTT (Invitrogen, CA, USA) to a formazan product. After appropriate incubation of cells with peptides, MTT was added to

each well and then incubated for 4 h at 37 °C. The reaction was terminated by the addition of 100 µl of SDS-HCL solution to each well. Following thorough mixing to dissolve the formazan product, the plates were read at 570 nm on a Spectramax M2 plate reader (Molecular Devices, Sunnyvale, CA). Assays were performed in replicate of three samples.

Apoptosis detection

The assay (cell death detection ELISA, Roche, Indianapolis, IN) is based on a quantitative sandwich enzyme immunoassay principle that determines the amount of mono-and oligonucleosomes, released in the cytoplasm by apoptotic endonucleases. Briefly, after treatments, cytosolic fraction of cell lysates were placed into streptavidin-coated microplate wells (Roche) and incubated with a mixture of biotinylated anti-histone antibodies and peroxidaselinked anti-DNA antibodies (Roche). Incubation and washing steps were performed according to manufacturer's instructions; peroxidase detection was determined spectrophotometrically with ABTS as substrate. The amount of colored product was measured at 405 nm on a Spectramax M2 plate reader.

Detection of Caspase-3 Activity

Cell were washed twice with PBS and lysed with cell lysis buffer (10 mM Tris-HCl, 10 mM NaH2PO4/NaHPO4, pH 7.5, 130 mM NaCl, 1% Triton -X-100, 10 mM NaN_3. The caspase-3 substrate used in this study, Ac-DEVD-AMC (Calbiochem/EMD Millipore, Billerica, MA), was prepared as a stock solution of 10 mm in water and stored at −20^{o}C. Prior to the assay, the caspase-3 substrate was diluted in 50 mM Tris‑HCl,

100 mM NaCl, 5 mM EDTA, 1 mM EGTA, 3 mM NaN_3, pH 7.3, yielding a final concentration of 25 μM Ac-DEVD-AMC in the assay. At the start of the proteolytic assay, 100 μl of cell lysate and 100 μl of substrate were added to a 96-well plate and fluorescence of the cleavage product was measured over time at 37°C in a Spectramax M2 plate reader, excitation wavelength 380 nm, emission 440 nm.

Western Blotting

Cells were lysed in a buffer containing 20 mM Tris-HCL, pH 7.4, 140 mM NaCl, 2 mM EDTA, 2 mM EGTA, 10% glycerol, 1% NP-40, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM phenylmthylsulfonyl fluoride. Samples were then subjected to centrifugation at 20,800 × *g* for 10 min. The supernatant was then transferred into Eppendorf tubes, and protein concentrations were determined using a Bio-Rad protein assay reagent. Protein was separated by SDS-polyacrylamide gel electrophoresis (4-20% gel, 25 μg of protein/sample) and then transferred to a nitrocellulose membrane and probed with monoclonal antibody raised against the phosphorylated or total forms of p38 (Cell Signaling Technology, Danvers, MA, USA). All blots were developed using Supersignal West Pico Chemiluminescent Substrate (Pierce).

Statistical analysis

All results were analyzed using two-way analysis of variance (ANOVA) with Bonferroni correction. P values of less than 0.05 were considered statistically significant. All graphs were generated using GraphPad Prism software (GraphPad Software, La Jolla, CA). Mean and SEM were calculated.

Figures

Figure 12: Dose response and time course of MDB cell death induced by DePrP, PrPY145Stop and PrP^{CWD}. Cells were treated with 50 μM (a), 200 μM (b) , or 400 μM (c) of the designated treatments and cell viability was assessed by MTT every 24 hours for 4 days. Data are expressed as a percentage of vehicle (buffer) treated samples, and all samples were conducted in triplicate. (d) Time course of MDB cell death induced by 200 uM as represented by plot summary data. Statistical significance was determined using two-way ANOVA with Bonferroni correction $P < 0.001$ versus control values. Different letters above bars denote statistical differences at the 0.001 level for the same time period.

Figure 12: Dose response and time course of MDB cell death induced by DePrP, PrPY145Stop and PrP^{CWD}

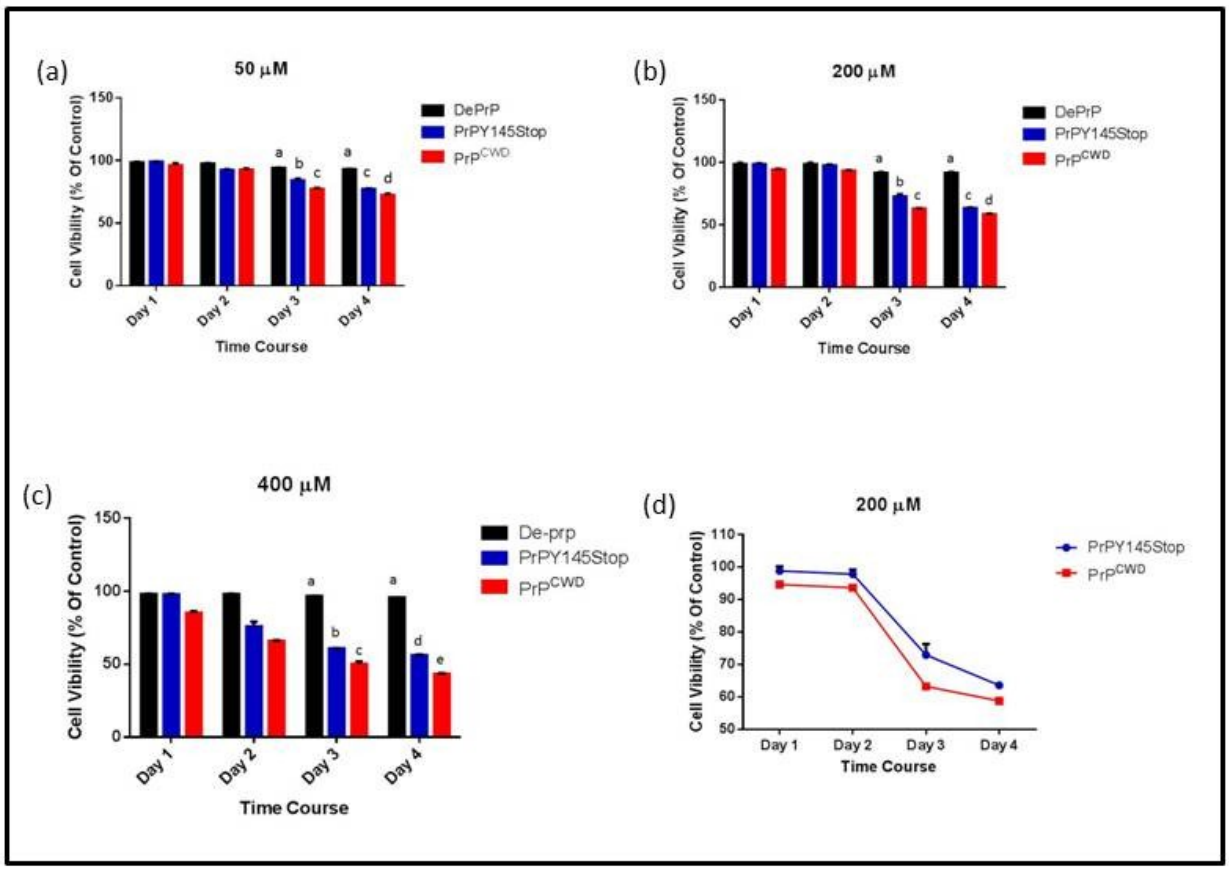


Figure 13: Induction of apoptosis in the MDB cell line by DePrP, PrPY145Stop and PrP^{CWD}. Cells were treated with 200 μM of the designated treatments and apoptosis was measured by an ELISA cell detection kit every day for 4 days. PrPY145Stop and PrP^{CWD} induced apoptosis that was significantly detectable after 2 days as compared with controls, which were samples treated with sample buffer. The values are expressed as a percentage of vehicle (buffer) treated control, and all samples were conducted in triplicate. $P < 0.01$ versus control values.

Figure 13: Induction of apoptosis in the MDB cell line by DePrP, PrPY145Stop and PrP^{CWD}

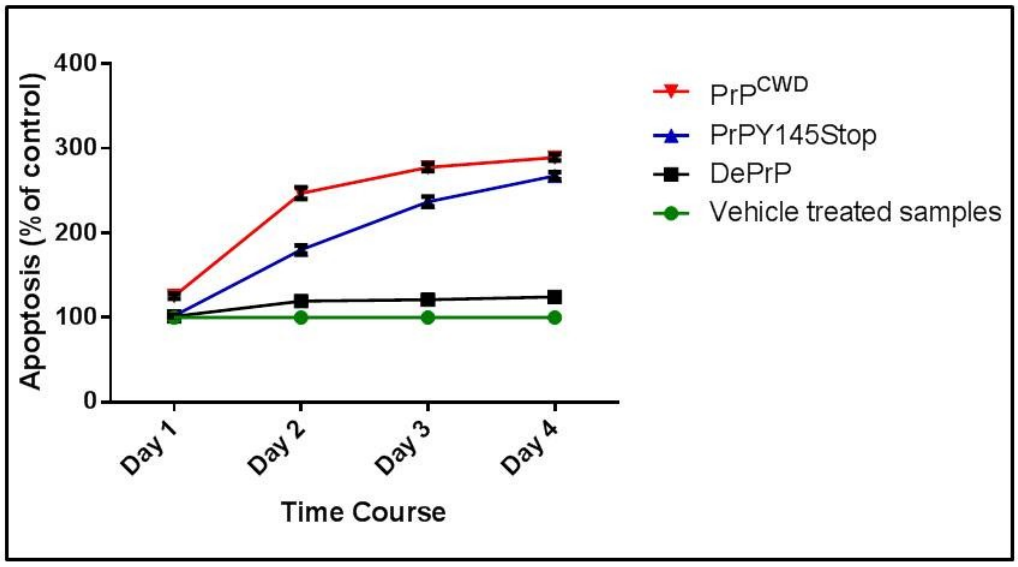


Figure 14: Caspase-3 activation as an early event in apoptosis induced by PrPY145Stop. (a) MDB cells were treated with PrPY145Stop (200 μM) for 4 hours. Caspase-3 activity was measured evaluating the cleavage of the fluorogenic substrate Ac-DEVD-AMC and compared to vehicle treated samples. Caspase-3 activity was detectable after 2 hours. (b) The induction of apoptosis was observed to be capsapse-3 dependent since it could be blocked by Z-VAD-FMK (Capsase inhibitor IV), a general caspase blocker, and Z-DEVD-FMK (Capsase inhibitor V), a more selective caspase-3 inhibitor. Data represent the mean and SEM of three experiments. $P < 0.01$.

Figure 14: Caspase-3 activation as an early event in apoptosis induced by PrPY145Stop

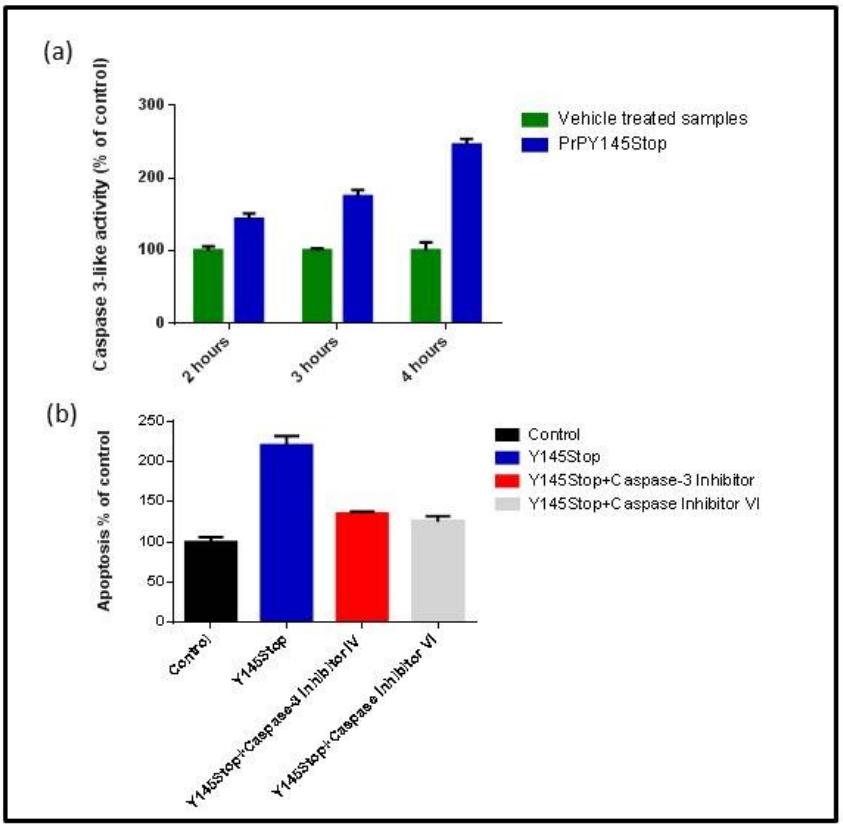
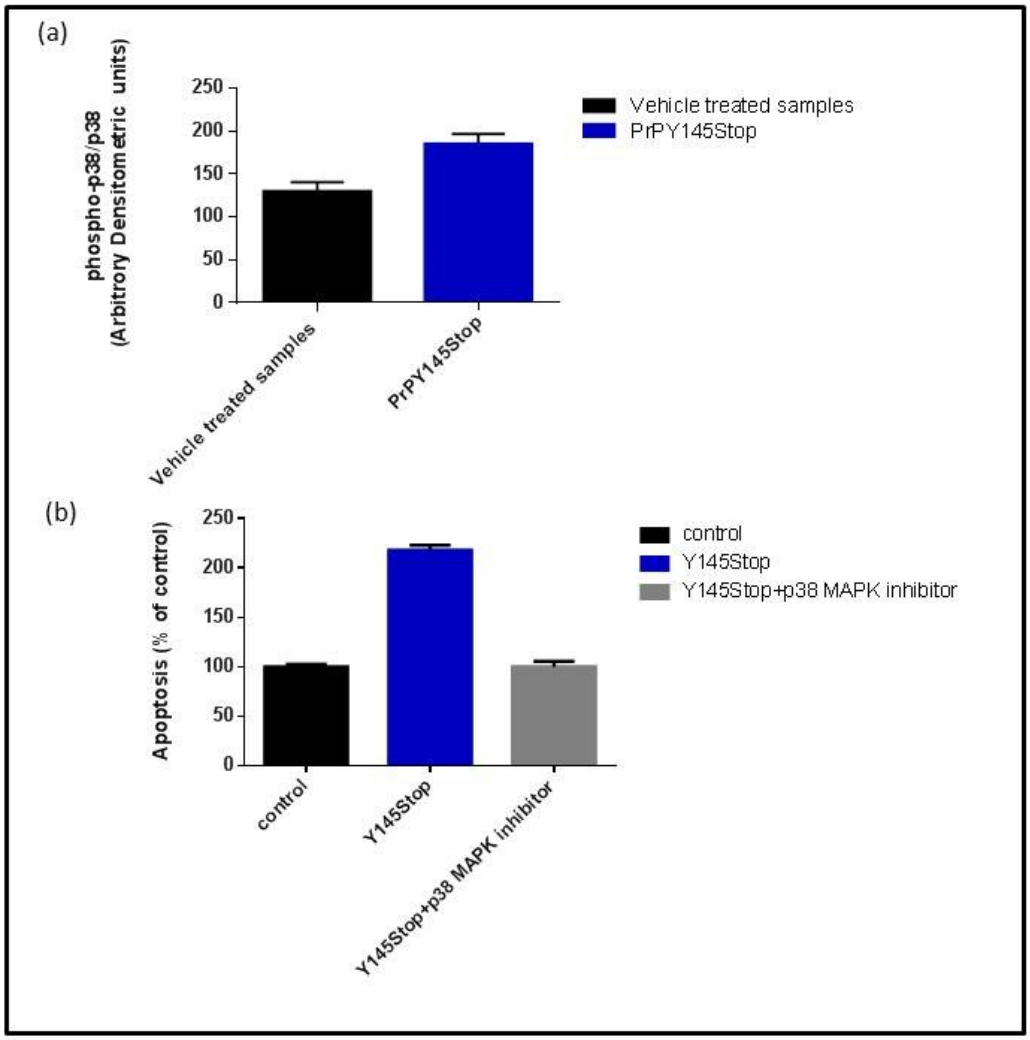


Figure 15: Role of p38 MAP kinase in apoptosis induction by PrPY145Stop. (a) Densitometric analysis of the phospho-p38/p38 ratio in vehicle- or PrPY145Stop-treated MDB cells. Densitometric data were obtained as optical density (OD) × mm2, and the ratios were expressed as a percentage of control (untreated) value. Each point represents the average of the results obtained from three independent Western blot experiments. (b) The induction of apoptosis was assayed in PrPY145-treated cells in the presence or absence of p38 blocker. The apoptosis effect was completely blocked by p38 MAP kinase as observed by ELISA after 3 days of treatment. All samples were conducted in triplicate. $P < 0.01$ versus control values.

Figure 15: Role of p38 MAP kinase in apoptosis induction by PrPY145Stop



References

1. Pan, K.M., et al., *Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins.* Proc Natl Acad Sci U S A, 1993. **90**(23): p. 10962-6.
2. Chiesa, R. and D.A. Harris, *Prion diseases: what is the neurotoxic molecule?* Neurobiol Dis, 2001. **8**(5): p. 743-63.
3. Hetz, C., et al., *Caspase-12 and endoplasmic reticulum stress mediate neurotoxicity of pathological prion protein.* EMBO J, 2003. **22**(20): p. 5435-45.
4. Marella, M., et al., *Pathological prion protein exposure switches on neuronal mitogen-activated protein kinase pathway resulting in microglia recruitment.* J Biol Chem, 2005. **280**(2): p. 1529-34.
5. Brown, D.R., et al., *The cellular prion protein binds copper in vivo.* Nature, 1997. **390**(6661): p. 684-7.
6. Wadsworth, J.D., et al., *Strain-specific prion-protein conformation determined by metal ions.* Nat Cell Biol, 1999. **1**(1): p. 55-9.
7. Martins, V.R., et al., *Insights into the physiological function of cellular prion protein.* Braz J Med Biol Res, 2001. **34**(5): p. 585-95.
8. Vassallo, N. and J. Herms, *Cellular prion protein function in copper homeostasis and redox signalling at the synapse.* J Neurochem, 2003. **86**(3): p. 538-44.
9. Wechselberger, C., et al., *The physiological functions of prion protein.* Exp Cell Res, 2002. **281**(1): p. 1-8.
10. Brown, D.R., R.S. Nicholas, and L. Canevari, *Lack of prion protein expression results in a neuronal phenotype sensitive to stress.* J Neurosci Res, 2002. **67**(2): p. 211-24.
11. Sakudo, A., et al., *Impairment of superoxide dismutase activation by N-terminally truncated prion protein (PrP) in PrP-deficient neuronal cell line.* Biochem Biophys Res Commun, 2003. **308**(3): p. 660-7.
12. Bounhar, Y., et al., *Prion protein protects human neurons against Bax-mediated apoptosis.* J Biol Chem, 2001. **276**(42): p. 39145-9.
13. Forloni, G., et al., *Neurotoxicity of a prion protein fragment.* Nature, 1993. **362**(6420): p. 543-6.
14. Corsaro, A., et al., *Prion protein fragment 106-126 induces a p38 MAP kinase-dependent apoptosis in SH-SY5Y neuroblastoma cells independently from the amyloid fibril formation.* Ann N Y Acad Sci, 2003. **1010**: p. 610-22.
15. Florio, T., et al., *Prion protein fragment 106-126 induces apoptotic cell death and impairment of L-type voltage-sensitive calcium channel activity in the GH3 cell line.* J Neurosci Res, 1998. **54**(3): p. 341-52.
16. Tagliavini, F., et al., *Synthetic peptides homologous to prion protein residues 106-147 form amyloid-like fibrils in vitro.* Proc Natl Acad Sci U S A, 1993. **90**(20): p. 9678-82.
17. De Gioia, L., et al., *Conformational polymorphism of the amyloidogenic and neurotoxic peptide homologous to residues 106-126 of the prion protein.* J Biol Chem, 1994. **269**(11): p. 7859-62.

18. Jobling, M.F., et al., *The hydrophobic core sequence modulates the neurotoxic and secondary structure properties of the prion peptide 106-126.* J Neurochem, 1999. **73**(4): p. 1557-65.
19. Thellung, S., et al., *Apoptotic cell death and impairment of L-type voltage-sensitive calcium channel activity in rat cerebellar granule cells treated with the prion protein fragment 106-126.* Neurobiol Dis, 2000. **7**(4): p. 299-309.
20. Thellung, S., et al., *Intracellular mechanisms mediating the neuronal death and astrogliosis induced by the prion protein fragment 106-126.* Int J Dev Neurosci, 2000. **18**(4-5): p. 481-92.
21. Thellung, S., et al., *p38 MAP kinase mediates the cell death induced by PrP106-126 in the SH-SY5Y neuroblastoma cells.* Neurobiol Dis, 2002. **9**(1): p. 69-81.
22. Fioriti, L., et al., *The neurotoxicity of prion protein (PrP) peptide 106-126 is independent of the expression level of PrP and is not mediated by abnormal PrP species.* Mol Cell Neurosci, 2005. **28**(1): p. 165-76.
23. Kitamoto, T., R. Iizuka, and J. Tateishi, *An amber mutation of prion protein in Gerstmann-Straussler syndrome with mutant PrP plaques.* Biochem Biophys Res Commun, 1993. **192**(2): p. 525-31.
24. Ghetti, B., et al., *Vascular variant of prion protein cerebral amyloidosis with tau-positive neurofibrillary tangles: the phenotype of the stop codon 145 mutation in PRNP.* Proc Natl Acad Sci U S A, 1996. **93**(2): p. 744-8.
25. Abdallah, A., et al., *Y145Stop is sufficient to induce de novo generation prions using protein misfolding cyclic amplification.* Prion, 2012. **6**(1).
26. Raymond, G.J., et al., *Inhibition of protease-resistant prion protein formation in a transformed deer cell line infected with chronic wasting disease.* J Virol, 2006. **80**(2): p. 596-604.
27. Vassalli, G., G. Milano, and T. Moccetti, *Role of Mitogen-Activated Protein Kinases in Myocardial Ischemia-Reperfusion Injury during Heart Transplantation.* J Transplant, 2012. **2012**: p. 928954.
28. Hope, J., et al., *Cytotoxicity of prion protein peptide (PrP106-126) differs in mechanism from the cytotoxic activity of the Alzheimer's disease amyloid peptide, A beta 25-35.* Neurodegeneration, 1996. **5**(1): p. 1-11.
29. O'Donovan, C.N., D. Tobin, and T.G. Cotter, *Prion protein fragment PrP-(106-126) induces apoptosis via mitochondrial disruption in human neuronal SH-SY5Y cells.* J Biol Chem, 2001. **276**(47): p. 43516-23.
30. Salgame, P., et al., *An ELISA for detection of apoptosis.* Nucleic Acids Res, 1997. **25**(3): p. 680-1.
31. Mielke, K. and T. Herdegen, *JNK and p38 stresskinases--degenerative effectors of signal-transduction-cascades in the nervous system.* Prog Neurobiol, 2000. **61**(1): p. 45-60.
32. Garrington, T.P. and G.L. Johnson, *Organization and regulation of mitogen-activated protein kinase signaling pathways.* Curr Opin Cell Biol, 1999. **11**(2): p. 211-8.
33. Sarkar, D., et al., *mda-7 (IL-24) Mediates selective apoptosis in human melanoma cells by inducing the coordinated overexpression of the GADD family of genes by means of p38 MAPK.* Proc Natl Acad Sci U S A, 2002. **99**(15): p. 10054-9.

34. Porras, A., et al., *P38 alpha mitogen-activated protein kinase sensitizes cells to apoptosis induced by different stimuli.* Mol Biol Cell, 2004. **15**(2): p. 922-33.
35. Park, J.M., et al., *Macrophage apoptosis by anthrax lethal factor through p38 MAP kinase inhibition.* Science, 2002. **297**(5589): p. 2048-51.
36. Juretic, N., et al., *ERK 1,2 and p38 pathways are involved in the proliferative stimuli mediated by urokinase in osteoblastic SaOS-2 cell line.* J Cell Biochem, 2001. **83**(1): p. 92-8.
37. Wang, P., et al., *Selection and characterization of DNA aptamers against PrP(Sc).* Exp Biol Med (Maywood), 2011. **236**(4): p. 466-76.

CONCLUDING REMARKS

The conversion of PrP^{C} to PrP^{Sc} is the most proximal event in the pathogenesis of transmissible spongiform encephalopathies (TSEs) or prion diseases. Hence, intervention strategies to mitigate these diseases usually depends on understanding PrP conversion and its relation to fundamental TSE disease parameters such as the transmission of infection, species barriers, the propagation of strains and neurotoxicity. Research indicates that PrP^{Sc} is an infectious agent that propagates itself by causing the conversion of PrP^{C} to PrP^{Sc}; however, the mechanisms by which this is achieved are not well understood. As an important step in unraveling the mechanism of prion conversion, It has been examined the role of N-terminal fragment of prion protein in seeding and misfolding of the mammalian prions.

Utilizing protein misfolding cyclic amplification (PMCA), it has been showed that the recombinant polypeptide corresponding to the Y145Stop of sheep and deer PRNP can be spontaneously converted to PK-resistant PrP^{Sc}. This conformational conversion was preceded by a lengthy lag phase ranging from 8-10 hrs and addition of a small quantity of spontaneously converted Y145Stop from sheep or deer to soluble protein monomer was able to seed the conversion and eliminate the lag phase. This was important to show that the N-terminal fragment by itself can follow the same seeding fashion as infectious prions. In contrast to PrP145Stop, none of the purified recombinant protein corresponding to full-length protein showed a propensity for spontaneous conversion to protease resistant isoforms indicating that C-terminal domain might protect the full molecule from triggering the conversion of PrP^{C} to PrP^{Sc} under the same conditions.

Furthermore, the efficiency of seeding between proteins derived from sheep and deer was examined. It was observed that seeded or spontaneously misfolded Y145Stop molecules can efficiently convert purified mammalian PrP^{C} into protease resistant isoforms. Identification that Y145Stop is sufficient to induce de novo generation of PrP^{Sc} significantly impacts our understanding of mechanism of prion conversion as a possible intermediate in the transition from normal cellular protein (termed PrP^{C}) into the pathogenic isoform.

The infectivity of PMCA-misfolded Y145Stop from sheep or deer in cell lines was examined next. It has been compared the capability of the different prion protein fragments to generate infectivity using MDB cells. Y145Stop induced prion conversion in 40% of the sublines, producing *de novo* infection as assayed by cell blotting. Infected susceptible sublines with partially purified PrP^{CWD} behaved similarly. The kinetics of PrP^{Sc} production showed increasing in the amounts of PrP^{Sc} after day 11 which indicate a de novo formation of PrP^{Sc}.

Finally, the molecular aspects of the cell death machinery triggered by Y145Stop using our cell culture model was studied. The toxicity of the PrPY145Stop peptide in cell culture has not been demonstrated previously. Therefore, the toxic effect of DeY145Stop in a brain-derived cell line, MDB, and the involvement of caspases and MAP kinases in this phenomenon were investigated. It has been showed that Y145Stop induced apoptosis in MDB cells through the activation of caspases 3 and p38 MAP kinase. Importantly, these data indicate that fibrillogenic properties of Y145Stop are associated with toxicity

through apoptotic signaling pathway. Therefore, Y145Stop can be a valuable model to study misfolding of mammalian prion and its toxic activity.

Taken together, data generated from these studies provide a new aspect in the prion conversion mechanism. Elucidating the role of the N-terminus in seeding and misfolding of mammalian prions has important implications for designing diagnostics and therapy of prion diseases as well as for understanding pathogenic mechanisms operative in interspecies transmission.

BIBLIOGRAPHY

Abdallah, A., P. Wang, et al. (2012). "Y145Stop is sufficient to induce de novo generation prions using protein misfolding cyclic amplification." Prion **6**(1).

Adams, D. H. (1998). "A dual pathway for the transmission of spongiform encephalopathy disease." Med Hypotheses **51**(1): 17-22.

Aguzzi, A. (1998). "Protein conformation dictates prion strain." Nat Med **4**(10): 1125-1126.

Aguzzi, A. and M. Heikenwalder (2003). "Prion diseases: Cannibals and garbage piles." Nature **423**(6936): 127-129.

Aguzzi, A. and M. Polymenidou (2004). "Mammalian prion biology: one century of evolving concepts." Cell **116**(2): 313-327.

Alexeeva, I., E. J. Elliott, et al. (2006). "Absence of Spiroplasma or other bacterial 16s rRNA genes in brain tissue of hamsters with scrapie." J Clin Microbiol **44**(1): 91-97.

Alper, T., W. A. Cramp, et al. (1967). "Does the agent of scrapie replicate without nucleic acid?" Nature **214**(5090): 764-766.

Alper, T., D. A. Haig, et al. (1966). "The exceptionally small size of the scrapie agent." Biochem Biophys Res Commun **22**(3): 278-284.

Apetri, A. C., D. L. Vanik, et al. (2005). "Polymorphism at residue 129 modulates the conformational conversion of the D178N variant of human prion protein 90-231." Biochemistry **44**(48): 15880-15888.

Atarashi, R., R. A. Moore, et al. (2007). "Ultrasensitive detection of scrapie prion protein using seeded conversion of recombinant prion protein." Nat Methods **4**(8): 645-650.

Bartz, J. C., M. L. Kramer, et al. (2007). "Prion interference is due to a reduction in strain-specific PrPSc levels." J Virol **81**(2): 689-697.

Baskakov, I. V. (2004). "Autocatalytic conversion of recombinant prion proteins displays a species barrier." J Biol Chem **279**(9): 7671-7677.

Basler, K., B. Oesch, et al. (1986). "Scrapie and cellular PrP isoforms are encoded by the same chromosomal gene." Cell **46**(3): 417-428.

Bastian, F. O., D. E. Sanders, et al. (2007). "Spiroplasma spp. from transmissible spongiform encephalopathy brains or ticks induce spongiform encephalopathy in ruminants." J Med Microbiol **56**(Pt 9): 1235-1242.

Belt, P. B., I. H. Muileman, et al. (1995). "Identification of five allelic variants of the sheep PrP gene and their association with natural scrapie." J Gen Virol **76 (Pt 3)**: 509-517.

Benestad, S. L., J. N. Arsac, et al. (2008). "Atypical/Nor98 scrapie: properties of the agent, genetics, and epidemiology." Vet Res **39**(4): 19.

Beringue, V., J. L. Vilotte, et al. (2008). "Prion agent diversity and species barrier." Vet Res **39**(4): 47.

Bessen, R. A., D. A. Kocisko, et al. (1995). "Non-genetic propagation of strain-specific properties of scrapie prion protein." Nature **375**(6533): 698-700.

Biacabe, A. G., J. L. Laplanche, et al. (2004). "Distinct molecular phenotypes in bovine prion diseases." EMBO Rep **5**(1): 110-115.

Bocharova, O. V., L. Breydo, et al. (2005). "In vitro conversion of full-length mammalian prion protein produces amyloid form with physical properties of PrP(Sc)." J Mol Biol **346**(2): 645-659.

Bolton, D. C., M. P. McKinley, et al. (1982). "Identification of a protein that purifies with the scrapie prion." Science **218**(4579): 1309-1311.

Borchelt, D. R., M. Scott, et al. (1990). "Scrapie and cellular prion proteins differ in their kinetics of synthesis and topology in cultured cells." J Cell Biol **110**(3): 743-752.

Bosque, P. J., C. Ryou, et al. (2002). "Prions in skeletal muscle." Proc Natl Acad Sci U S A **99**(6): 3812-3817.

Bounhar, Y., Y. Zhang, et al. (2001). "Prion protein protects human neurons against Bax-mediated apoptosis." J Biol Chem **276**(42): 39145-39149.

Brandner, S., S. Isenmann, et al. (1998). "Identification of the end stage of scrapie using infected neural grafts." Brain Pathol **8**(1): 19-27.

Brandner, S., S. Isenmann, et al. (1996). "Normal host prion protein necessary for scrapie-induced neurotoxicity." Nature **379**(6563): 339-343.

Brockes, J. P. (1999). "Topics in prion cell biology." Curr Opin Neurobiol **9**(5): 571-577.

Brown, D. R., R. S. Nicholas, et al. (2002). "Lack of prion protein expression results in a neuronal phenotype sensitive to stress." J Neurosci Res **67**(2): 211-224.

Brown, D. R., K. Qin, et al. (1997). "The cellular prion protein binds copper in vivo." Nature **390**(6661): 684-687.

Bruce, M., A. Chree, et al. (1994). "Transmission of bovine spongiform encephalopathy and scrapie to mice: strain variation and the species barrier." Philos Trans R Soc Lond B Biol Sci **343**(1306): 405-411.

Bruce, M. E. (1993). "Scrapie strain variation and mutation." Br Med Bull **49**(4): 822-838.

Bruce, M. E. (2003). "TSE strain variation." Br Med Bull **66**: 99-108.

Bruce, M. E., A. Boyle, et al. (2002). "Strain characterization of natural sheep scrapie and comparison with BSE." J Gen Virol **83**(Pt 3): 695-704.

Bruce, M. E. and A. G. Dickinson (1987). "Biological evidence that scrapie agent has an independent genome." J Gen Virol **68 (Pt 1)**: 79-89.

Bueler, H., A. Aguzzi, et al. (1993). "Mice devoid of PrP are resistant to scrapie." Cell **73**(7): 1339-1347.

Bueler, H., M. Fischer, et al. (1992). "Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein." Nature **356**(6370): 577-582.

Buschmann, A., A. G. Biacabe, et al. (2004). "Atypical scrapie cases in Germany and France are identified by discrepant reaction patterns in BSE rapid tests." J Virol Methods **117**(1): 27-36.

Casalone, C., G. Zanusso, et al. (2004). "Identification of a second bovine amyloidotic spongiform encephalopathy: molecular similarities with sporadic Creutzfeldt-Jakob disease." Proc Natl Acad Sci U S A **101**(9): 3065-3070.

Castilla, J., P. Saa, et al. (2005). "In vitro generation of infectious scrapie prions." Cell **121**(2): 195-206.

Castilla, J., P. Saa, et al. (2006). "Protein misfolding cyclic amplification for diagnosis and prion propagation studies." Methods Enzymol **412**: 3-21.

Chabry, J., S. A. Priola, et al. (1999). "Species-independent inhibition of abnormal prion protein (PrP) formation by a peptide containing a conserved PrP sequence." J Virol **73**(8): 6245-6250.

Chesebro, B. (1998). "BSE and prions: uncertainties about the agent." Science **279**(5347): 42-43.

Chesebro, B., R. Race, et al. (1985). "Identification of scrapie prion protein-specific mRNA in scrapie-infected and uninfected brain." Nature **315**(6017): 331-333.

Chiesa, R. and D. A. Harris (2001). "Prion diseases: what is the neurotoxic molecule?" Neurobiol Dis **8**(5): 743-763.

Cho, H. J. (1976). "Is the scrapie agent a virus?" Nature **262**(5567): 411-412.

Chretien, F., A. Dorandeu, et al. (1999). "[A process of programmed cell death as a mechanisms of neuronal death in prion diseases]." Clin Exp Pathol **47**(3-4): 181-191.

Collinge, J. (2001). "Prion diseases of humans and animals: their causes and molecular basis." Annu Rev Neurosci **24**: 519-550.

Collinge, J., K. C. Sidle, et al. (1996). "Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD." Nature **383**(6602): 685-690.

Collinge, J., M. A. Whittington, et al. (1994). "Prion protein is necessary for normal synaptic function." Nature **370**(6487): 295-297.

Come, J. H., P. E. Fraser, et al. (1993). "A kinetic model for amyloid formation in the prion diseases: importance of seeding." Proc Natl Acad Sci U S A **90**(13): 5959-5963.

Cordeiro, Y., F. Machado, et al. (2001). "DNA converts cellular prion protein into the beta-sheet conformation and inhibits prion peptide aggregation." J Biol Chem **276**(52): 49400-49409.

Corsaro, A., S. Thellung, et al. (2003). "Prion protein fragment 106-126 induces a p38 MAP kinase-dependent apoptosis in SH-SY5Y neuroblastoma cells independently from the amyloid fibril formation." Ann N Y Acad Sci **1010**: 610-622.

Cullie, J. C., P.L. (1939). "Experimental transmission of trembling to the goat." C. R. Seances Acad. Sci. **208**: 1058-1160.

De Gioia, L., C. Selvaggini, et al. (1994). "Conformational polymorphism of the amyloidogenic and neurotoxic peptide homologous to residues 106-126 of the prion protein." J Biol Chem **269**(11): 7859-7862.

DebBurman, S. K., G. J. Raymond, et al. (1997). "Chaperone-supervised conversion of prion protein to its protease-resistant form." Proc Natl Acad Sci U S A **94**(25): 13938-13943.

Deleault, N. R., J. C. Geoghegan, et al. (2005). "Protease-resistant prion protein amplification reconstituted with partially purified substrates and synthetic polyanions." J Biol Chem **280**(29): 26873-26879.

Deleault, N. R., B. T. Harris, et al. (2007). "Formation of native prions from minimal components in vitro." Proc Natl Acad Sci U S A **104**(23): 9741-9746.

Deleault, N. R., R. W. Lucassen, et al. (2003). "RNA molecules stimulate prion protein conversion." Nature **425**(6959): 717-720.

Dickinson, A. G. and G. W. Outram (1988). "Genetic aspects of unconventional virus infections: the basis of the virino hypothesis." Ciba Found Symp **135**: 63-83.

Dorandeu, A., L. Wingertsmann, et al. (1998). "Neuronal apoptosis in fatal familial insomnia." Brain Pathol **8**(3): 531-537.

Durig, J., A. Giese, et al. (2000). "Differential constitutive and activation-dependent expression of prion protein in human peripheral blood leucocytes." Br J Haematol **108**(3): 488-495.

Elsen, J. M., Y. Amigues, et al. (1999). "Genetic susceptibility and transmission factors in scrapie: detailed analysis of an epidemic in a closed flock of Romanov." Arch Virol **144**(3): 431-445.

Enari, M., E. Flechsig, et al. (2001). "Scrapie prion protein accumulation by scrapie-infected neuroblastoma cells abrogated by exposure to a prion protein antibody." Proc Natl Acad Sci U S A **98**(16): 9295-9299.

Fioriti, L., E. Quaglio, et al. (2005). "The neurotoxicity of prion protein (PrP) peptide 106-126 is independent of the expression level of PrP and is not mediated by abnormal PrP species." Mol Cell Neurosci **28**(1): 165-176.

Florio, T., S. Thellung, et al. (1998). "Prion protein fragment 106-126 induces apoptotic cell death and impairment of L-type voltage-sensitive calcium channel activity in the GH3 cell line." J Neurosci Res **54**(3): 341-352.

Forloni, G., N. Angeretti, et al. (1993). "Neurotoxicity of a prion protein fragment." Nature **362**(6420): 543-546.

Gabizon, R., M. P. McKinley, et al. (1988). "Immunoaffinity purification and neutralization of scrapie prion infectivity." Proc Natl Acad Sci U S A **85**(18): 6617-6621.

Gabizon, R. and S. B. Prusiner (1990). "Prion liposomes." Biochem J **266**(1): 1-14.

Gabus, C., E. Derrington, et al. (2001). "The prion protein has RNA binding and chaperoning properties characteristic of nucleocapsid protein NCP7 of HIV-1." J Biol Chem **276**(22): 19301-19309.

Gajdusek, D. C. (1988). "Transmissible and non-transmissible amyloidoses: autocatalytic post-translational conversion of host precursor proteins to beta-pleated sheet configurations." J Neuroimmunol **20**(2-3): 95-110.

Gajdusek, D. C., C. J. Gibbs, et al. (1966). "Experimental transmission of a Kuru-like syndrome to chimpanzees." Nature **209**(5025): 794-796.

Garrington, T. P. and G. L. Johnson (1999). "Organization and regulation of mitogen-activated protein kinase signaling pathways." Curr Opin Cell Biol **11**(2): 211-218.

Gasset, M., M. A. Baldwin, et al. (1992). "Predicted alpha-helical regions of the prion protein when synthesized as peptides form amyloid." Proc Natl Acad Sci U S A **89**(22): 10940-10944.

Ghetti, B., P. Piccardo, et al. (1996). "Vascular variant of prion protein cerebral amyloidosis with tau-positive neurofibrillary tangles: the phenotype of the stop codon 145 mutation in PRNP." Proc Natl Acad Sci U S A **93**(2): 744-748.

Gibbs, C. J., Jr., D. C. Gajdusek, et al. (1968). "Creutzfeldt-Jakob disease (spongiform encephalopathy): transmission to the chimpanzee." Science **161**(3839): 388-389.

Giese, A., M. H. Groschup, et al. (1995). "Neuronal cell death in scrapie-infected mice is due to apoptosis." Brain Pathol **5**(3): 213-221.

Goldmann, W. (2008). "PrP genetics in ruminant transmissible spongiform encephalopathies." Vet Res **39**(4): 30.

Goldmann, W., N. Hunter, et al. (1990). "Two alleles of a neural protein gene linked to scrapie in sheep." Proc Natl Acad Sci U S A **87**(7): 2476-2480.

Goldmann, W., T. Martin, et al. (1996). "Novel polymorphisms in the caprine PrP gene: a codon 142 mutation associated with scrapie incubation period." J Gen Virol **77 (Pt 11)**: 2885-2891.

Gordon, W. S. (1946). "Advances in veterinary research." Vet Rec **58**(47): 516-525.

Gossert, A. D., S. Bonjour, et al. (2005). "Prion protein NMR structures of elk and of mouse/elk hybrids." Proc Natl Acad Sci U S A **102**(3): 646-650.

Gray, F., H. Adle-Biassette, et al. (1999). "[Neuronal apoptosis in human prion diseases]." Bull Acad Natl Med **183**(2): 305-320; discussion 320-301.

Gray, F., F. Chretien, et al. (1999). "Neuronal apoptosis in Creutzfeldt-Jakob disease." J Neuropathol Exp Neurol **58**(4): 321-328.

Griffith, J. S. (1967). "Self-replication and scrapie." Nature **215**(5105): 1043-1044.

Hay, B., R. A. Barry, et al. (1987). "Biogenesis and transmembrane orientation of the cellular isoform of the scrapie prion protein [published errratum appears in Mol Cell Biol 1987 May;7(5):2035]." Mol Cell Biol **7**(2): 914-920.

Hegde, R. S., J. A. Mastrianni, et al. (1998). "A transmembrane form of the prion protein in neurodegenerative disease." Science **279**(5352): 827-834.

Hegde, R. S., S. Voigt, et al. (1998). "Regulation of protein topology by trans-acting factors at the endoplasmic reticulum." Mol Cell **2**(1): 85-91.

Hetz, C., M. Russelakis-Carneiro, et al. (2003). "Caspase-12 and endoplasmic reticulum stress mediate neurotoxicity of pathological prion protein." EMBO J **22**(20): 5435-5445.

Hill, A. F., M. Antoniou, et al. (1999). "Protease-resistant prion protein produced in vitro lacks detectable infectivity." J Gen Virol **80 (Pt 1)**: 11-14.

Hill, A. F., S. Joiner, et al. (2003). "Molecular classification of sporadic Creutzfeldt-Jakob disease." Brain **126**(Pt 6): 1333-1346.

Hope, J., M. S. Shearman, et al. (1996). "Cytotoxicity of prion protein peptide (PrP106-126) differs in mechanism from the cytotoxic activity of the Alzheimer's disease amyloid peptide, A beta 25-35." Neurodegeneration **5**(1): 1-11.

Hornemann, S., C. Korth, et al. (1997). "Recombinant full-length murine prion protein, mPrP(23-231): purification and spectroscopic characterization." FEBS Lett **413**(2): 277-281.

Hsiao, K., H. F. Baker, et al. (1989). "Linkage of a prion protein missense variant to Gerstmann-Straussler syndrome." Nature **338**(6213): 342-345.

Hsiao, K. K., M. Scott, et al. (1990). "Spontaneous neurodegeneration in transgenic mice with mutant prion protein." Science **250**(4987): 1587-1590.

Hutter, G., F. L. Heppner, et al. (2003). "No superoxide dismutase activity of cellular prion protein in vivo." Biol Chem **384**(9): 1279-1285.

Jesionek-Kupnicka, D., J. Buczynski, et al. (1997). "Programmed cell death (apoptosis) in Alzheimer's disease and Creutzfeldt-Jakob disease." Folia Neuropathol **35**(4): 233-235.

Jesionek-Kupnicka, D., R. Kordek, et al. (2001). "Apoptosis in relation to neuronal loss in experimental Creutzfeldt-Jakob disease in mice." Acta Neurobiol Exp (Wars) **61**(1): 13-19.

Jobling, M. F., L. R. Stewart, et al. (1999). "The hydrophobic core sequence modulates the neurotoxic and secondary structure properties of the prion peptide 106-126." J Neurochem **73**(4): 1557-1565.

Jones, E. M. and W. K. Surewicz (2005). "Fibril conformation as the basis of species- and strain-dependent seeding specificity of mammalian prion amyloids." Cell **121**(1): 63-72.

Juretic, N., J. F. Santibanez, et al. (2001). "ERK 1,2 and p38 pathways are involved in the proliferative stimuli mediated by urokinase in osteoblastic SaOS-2 cell line." J Cell Biochem **83**(1): 92-98.

Kaneko, K., H. L. Ball, et al. (2000). "A synthetic peptide initiates Gerstmann-Straussler-Scheinker (GSS) disease in transgenic mice." J Mol Biol **295**(4): 997-1007.

Kim, J. I., I. Cali, et al. (2010). "Mammalian prions generated from bacterially expressed prion protein in the absence of any mammalian cofactors." J Biol Chem **285**(19): 14083-14087.

Kimberlin, R. H. and C. A. Walker (1978). "Evidence that the transmission of one source of scrapie agent to hamsters involves separation of agent strains from a mixture." J Gen Virol **39**(3): 487-496.

Kitamoto, T., R. Iizuka, et al. (1993). "An amber mutation of prion protein in Gerstmann-Straussler syndrome with mutant PrP plaques." Biochem Biophys Res Commun **192**(2): 525-531.

Kitamoto, T. and J. Tateishi (1996). "Human prion disease and human prion protein disease." Curr Top Microbiol Immunol **207**: 27-34.

Kocisko, D. A., J. H. Come, et al. (1994). "Cell-free formation of protease-resistant prion protein." Nature **370**(6489): 471-474.

Kocisko, D. A., S. A. Priola, et al. (1995). "Species specificity in the cell-free conversion of prion protein to protease-resistant forms: a model for the scrapie species barrier." Proc Natl Acad Sci U S A **92**(9): 3923-3927.

Kundu, B., N. R. Maiti, et al. (2003). "Nucleation-dependent conformational conversion of the Y145Stop variant of human prion protein: structural clues for prion propagation." Proc Natl Acad Sci U S A **100**(21): 12069-12074.

Kurschner, C. and J. I. Morgan (1995). "The cellular prion protein (PrP) selectively binds to Bcl-2 in the yeast two-hybrid system." Brain Res Mol Brain Res **30**(1): 165-168.

Kurschner, C. and J. I. Morgan (1996). "Analysis of interaction sites in homo- and heteromeric complexes containing Bcl-2 family members and the cellular prion protein." Brain Res Mol Brain Res **37**(1-2): 249-258.

Kuwahara, C., A. M. Takeuchi, et al. (1999). "Prions prevent neuronal cell-line death." Nature **400**(6741): 225-226.

Leclerc, E., H. Serban, et al. (2006). "Copper induces conformational changes in the N-terminal part of cell-surface PrPC." Arch Virol **151**(11): 2103-2109.

Lee, S. and D. Eisenberg (2003). "Seeded conversion of recombinant prion protein to a disulfide-bonded oligomer by a reduction-oxidation process." Nat Struct Biol **10**(9): 725-730.

Legname, G., I. V. Baskakov, et al. (2004). "Synthetic mammalian prions." Science **305**(5684): 673-676.

Lucassen, P. J., A. Williams, et al. (1995). "Detection of apoptosis in murine scrapie." Neurosci Lett **198**(3): 185-188.

Makarava, N., G. G. Kovacs, et al. (2010). "Recombinant prion protein induces a new transmissible prion disease in wild-type animals." Acta Neuropathol **119**(2): 177-187.

Malaga-Trillo, E., G. P. Solis, et al. (2009). "Regulation of embryonic cell adhesion by the prion protein." PLoS Biol **7**(3): e55.

Mallucci, G. R., S. Ratte, et al. (2002). "Post-natal knockout of prion protein alters hippocampal CA1 properties, but does not result in neurodegeneration." EMBO J **21**(3): 202-210.

Manuelidis, L. (1998). "Vaccination with an attenuated Creutzfeldt-Jakob disease strain prevents expression of a virulent agent." Proc Natl Acad Sci U S A **95**(5): 2520-2525.

Marella, M., C. Gaggioli, et al. (2005). "Pathological prion protein exposure switches on neuronal mitogen-activated protein kinase pathway resulting in microglia recruitment." J Biol Chem **280**(2): 1529-1534.

Martins, V. R., A. F. Mercadante, et al. (2001). "Insights into the physiological function of cellular prion protein." Braz J Med Biol Res **34**(5): 585-595.

Masters, C. L., D. C. Gajdusek, et al. (1981). "Creutzfeldt-Jakob disease virus isolations from the Gerstmann-Straussler syndrome with an analysis of the various forms of amyloid plaque deposition in the virus-induced spongiform encephalopathies." Brain **104**(3): 559-588.

McCulloch, L., K. L. Brown, et al. (2011). "Follicular dendritic cell-specific prion protein (PrP) expression alone is sufficient to sustain prion infection in the spleen." PLoS Pathog **7**(12): e1002402.

Meier, P., N. Genoud, et al. (2003). "Soluble dimeric prion protein binds PrP(Sc) in vivo and antagonizes prion disease." Cell **113**(1): 49-60.

Mielke, K. and T. Herdegen (2000). "JNK and p38 stresskinases--degenerative effectors of signal-transduction-cascades in the nervous system." Prog Neurobiol **61**(1): 45-60.

Moore, R. A., C. Herzog, et al. (2006). "Octapeptide repeat insertions increase the rate of protease-resistant prion protein formation." Protein Sci **15**(3): 609-619.

Morillas, M., D. L. Vanik, et al. (2001). "On the mechanism of alpha-helix to beta-sheet transition in the recombinant prion protein." Biochemistry **40**(23): 6982-6987.

Moum, T., I. Olsaker, et al. (2005). "Polymorphisms at codons 141 and 154 in the ovine prion protein gene are associated with scrapie Nor98 cases." J Gen Virol **86**(Pt 1): 231-235.

Muramoto, T., M. Scott, et al. (1996). "Recombinant scrapie-like prion protein of 106 amino acids is soluble." Proc Natl Acad Sci U S A **93**(26): 15457-15462.

Nandi, P. K. (1997). "Interaction of prion peptide HuPrP106-126 with nucleic acid." Arch Virol **142**(12): 2537-2545.

Nishida, N., P. Tremblay, et al. (1999). "A mouse prion protein transgene rescues mice deficient for the prion protein gene from purkinje cell degeneration and demyelination." Lab Invest **79**(6): 689-697.

O'Donovan, C. N., D. Tobin, et al. (2001). "Prion protein fragment PrP-(106-126) induces apoptosis via mitochondrial disruption in human neuronal SH-SY5Y cells." J Biol Chem **276**(47): 43516-43523.

Oesch, B., D. Westaway, et al. (1985). "A cellular gene encodes scrapie PrP 27-30 protein." Cell **40**(4): 735-746.

Orge, L., A. Galo, et al. (2004). "Identification of putative atypical scrapie in sheep in Portugal." J Gen Virol **85**(Pt 11): 3487-3491.

Pan, K. M., M. Baldwin, et al. (1993). "Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins." Proc Natl Acad Sci U S A **90**(23): 10962-10966.

Park, J. M., F. R. Greten, et al. (2002). "Macrophage apoptosis by anthrax lethal factor through p38 MAP kinase inhibition." Science **297**(5589): 2048-2051.

Pattison, I. H. and G. C. Millson (1961). "Scrapie produced experimentally in goats with special reference to the clinical syndrome." J Comp Pathol **71**: 101-109.

Peretz, D., R. A. Williamson, et al. (2001). "Antibodies inhibit prion propagation and clear cell cultures of prion infectivity." Nature **412**(6848): 739-743.

Perini, F., R. Vidal, et al. (1996). "PrP27-30 is a normal soluble prion protein fragment released by human platelets." Biochem Biophys Res Commun **223**(3): 572-577.

Polymenidou, M., K. Stoeck, et al. (2005). "Coexistence of multiple PrPSc types in individuals with Creutzfeldt-Jakob disease." Lancet Neurol **4**(12): 805-814.

Porras, A., S. Zuluaga, et al. (2004). "P38 alpha mitogen-activated protein kinase sensitizes cells to apoptosis induced by different stimuli." Mol Biol Cell **15**(2): 922-933.

Priola, S. A. and B. Chesebro (1995). "A single hamster PrP amino acid blocks conversion to protease-resistant PrP in scrapie-infected mouse neuroblastoma cells." J Virol **69**(12): 7754-7758.

Prusiner, S. B. (1982). "Novel proteinaceous infectious particles cause scrapie." Science **216**(4542): 136-144.

Prusiner, S. B. (1989). "Scrapie prions." Annu Rev Microbiol **43**: 345-374.

Prusiner, S. B. (1991). "Molecular biology of prion diseases." Science **252**(5012): 1515-1522.

Prusiner, S. B. (1997). "Prion diseases and the BSE crisis." Science **278**(5336): 245-251.

Prusiner, S. B. (1998). "Prions." Proc Natl Acad Sci U S A **95**(23): 13363-13383.

Prusiner, S. B. and S. J. DeArmond (1991). "Molecular biology and pathology of scrapie and the prion diseases of humans." Brain Pathol **1**(4): 297-310.

Prusiner, S. B. and S. J. DeArmond (1994). "Prion diseases and neurodegeneration." Annu Rev Neurosci **17**: 311-339.

Prusiner, S. B., D. F. Groth, et al. (1984). "Purification and structural studies of a major scrapie prion protein." Cell **38**(1): 127-134.

Quaglio, E., R. Chiesa, et al. (2001). "Copper converts the cellular prion protein into a protease-resistant species that is distinct from the scrapie isoform." J Biol Chem **276**(14): 11432-11438.

Radovanovic, I., N. Braun, et al. (2005). "Truncated prion protein and Doppel are myelinotoxic in the absence of oligodendrocytic PrPC." J Neurosci **25**(19): 4879-4888.

Ramasamy, I., M. Law, et al. (2003). "Organ distribution of prion proteins in variant Creutzfeldt-Jakob disease." Lancet Infect Dis **3**(4): 214-222.

Raymond, G. J., A. Bossers, et al. (2000). "Evidence of a molecular barrier limiting susceptibility of humans, cattle and sheep to chronic wasting disease." EMBO J **19**(17): 4425-4430.

Raymond, G. J., E. A. Olsen, et al. (2006). "Inhibition of protease-resistant prion protein formation in a transformed deer cell line infected with chronic wasting disease." J Virol **80**(2): 596-604.

Riek, R., S. Hornemann, et al. (1996). "NMR structure of the mouse prion protein domain PrP(121-231)." Nature **382**(6587): 180-182.

Riek, R., S. Hornemann, et al. (1997). "NMR characterization of the full-length recombinant murine prion protein, mPrP(23-231)." FEBS Lett **413**(2): 282-288.

Roucou, X., P. N. Giannopoulos, et al. (2005). "Cellular prion protein inhibits proapoptotic Bax conformational change in human neurons and in breast carcinoma MCF-7 cells." Cell Death Differ **12**(7): 783-795.

Russelakis-Carneiro, M., G. P. Saborio, et al. (2002). "Changes in the glycosylation pattern of prion protein in murine scrapie. Implications for the mechanism of neurodegeneration in prion diseases." J Biol Chem **277**(39): 36872-36877.

Saa, P., J. Castilla, et al. (2006). "Ultra-efficient replication of infectious prions by automated protein misfolding cyclic amplification." J Biol Chem **281**(46): 35245-35252.

Saborio, G. P., B. Permanne, et al. (2001). "Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding." Nature **411**(6839): 810-813.

Safar, J., P. P. Roller, et al. (1993). "Thermal stability and conformational transitions of scrapie amyloid (prion) protein correlate with infectivity." Protein Sci **2**(12): 2206-2216.

Safar, J. G., K. Kellings, et al. (2005). "Search for a prion-specific nucleic acid." J Virol **79**(16): 10796-10806.

Sakudo, A., D. C. Lee, et al. (2003). "Impairment of superoxide dismutase activation by N-terminally truncated prion protein (PrP) in PrP-deficient neuronal cell line." Biochem Biophys Res Commun **308**(3): 660-667.

Salgame, P., A. S. Varadhachary, et al. (1997). "An ELISA for detection of apoptosis." Nucleic Acids Res **25**(3): 680-681.

Sarkar, D., Z. Z. Su, et al. (2002). "mda-7 (IL-24) Mediates selective apoptosis in human melanoma cells by inducing the coordinated overexpression of the GADD family of genes by means of p38 MAPK." Proc Natl Acad Sci U S A **99**(15): 10054-10059.

Scott, M., D. Foster, et al. (1989). "Transgenic mice expressing hamster prion protein produce species-specific scrapie infectivity and amyloid plaques." Cell **59**(5): 847-857.

Shmerling, D., I. Hegyi, et al. (1998). "Expression of amino-terminally truncated PrP in the mouse leading to ataxia and specific cerebellar lesions." Cell **93**(2): 203-214.

Sigurdson, C. J., G. Manco, et al. (2006). "Strain fidelity of chronic wasting disease upon murine adaptation." J Virol **80**(24): 12303-12311.

Silveira, J. R., G. J. Raymond, et al. (2005). "The most infectious prion protein particles." Nature **437**(7056): 257-261.

Solforosi, L., J. R. Criado, et al. (2004). "Cross-linking cellular prion protein triggers neuronal apoptosis in vivo." Science **303**(5663): 1514-1516.

Stahl, N., D. R. Borchelt, et al. (1987). "Scrapie prion protein contains a phosphatidylinositol glycolipid." Cell **51**(2): 229-240.

Supattapone, S., P. Bosque, et al. (1999). "Prion protein of 106 residues creates an artifical transmission barrier for prion replication in transgenic mice." Cell **96**(6): 869-878.

Supattapone, S., E. Bouzamondo, et al. (2001). "A protease-resistant 61-residue prion peptide causes neurodegeneration in transgenic mice." Mol Cell Biol **21**(7): 2608-2616.

Tagliavini, F., F. Prelli, et al. (1993). "Synthetic peptides homologous to prion protein residues 106-147 form amyloid-like fibrils in vitro." Proc Natl Acad Sci U S A **90**(20): 9678-9682.

Takemura, K., P. Wang, et al. (2006). "DNA aptamers that bind to PrP(C) and not PrP(Sc) show sequence and structure specificity." Exp Biol Med (Maywood) **231**(2): 204-214.

Theil, D., R. Fatzer, et al. (1999). "Nuclear DNA fragmentation and immune reactivity in bovine spongiform encephalopathy." J Comp Pathol **121**(4): 357-367.

Thellung, S., T. Florio, et al. (2000). "Intracellular mechanisms mediating the neuronal death and astrogliosis induced by the prion protein fragment 106-126." Int J Dev Neurosci **18**(4-5): 481-492.

Thellung, S., V. Villa, et al. (2002). "p38 MAP kinase mediates the cell death induced by PrP106-126 in the SH-SY5Y neuroblastoma cells." Neurobiol Dis **9**(1): 69-81.

Tobler, I., S. E. Gaus, et al. (1996). "Altered circadian activity rhythms and sleep in mice devoid of prion protein." Nature **380**(6575): 639-642.

Tremblay, P., H. L. Ball, et al. (2004). "Mutant PrPSc conformers induced by a synthetic peptide and several prion strains." J Virol **78**(4): 2088-2099.

Vanik, D. L., K. A. Surewicz, et al. (2004). "Molecular basis of barriers for interspecies transmissibility of mammalian prions." Mol Cell **14**(1): 139-145.

Vassalli, G., G. Milano, et al. (2012). "Role of Mitogen-Activated Protein Kinases in Myocardial Ischemia-Reperfusion Injury during Heart Transplantation." J Transplant **2012**: 928954.

Vassallo, N. and J. Herms (2003). "Cellular prion protein function in copper homeostasis and redox signalling at the synapse." J Neurochem **86**(3): 538-544.

Viles, J. H., F. E. Cohen, et al. (1999). "Copper binding to the prion protein: structural implications of four identical cooperative binding sites." Proc Natl Acad Sci U S A **96**(5): 2042-2047.

Vorberg, I. and S. A. Priola (2002). "Molecular basis of scrapie strain glycoform variation." J Biol Chem **277**(39): 36775-36781.

Wadsworth, J. D., E. A. Asante, et al. (2004). "Human prion protein with valine 129 prevents expression of variant CJD phenotype." Science **306**(5702): 1793-1796.

Wadsworth, J. D., A. F. Hill, et al. (1999). "Strain-specific prion-protein conformation determined by metal ions." Nat Cell Biol **1**(1): 55-59.

Wang, F., X. Wang, et al. (2010). "Generating a prion with bacterially expressed recombinant prion protein." Science **327**(5969): 1132-1135.

Wang, P., K. L. Hatcher, et al. (2011). "Selection and characterization of DNA aptamers against PrP(Sc)." Exp Biol Med (Maywood) **236**(4): 466-476.

Wechselberger, C., S. Wurm, et al. (2002). "The physiological functions of prion protein." Exp Cell Res **281**(1): 1-8.

Weissman, J. S. and J. K. Hood (2001). "Prion diseases. A rogue protein." Lancet **358 Suppl**: S53.

Westergard, L., H. M. Christensen, et al. (2007). "The cellular prion protein (PrP(C)): its physiological function and role in disease." Biochim Biophys Acta **1772**(6): 629-644.

Wong, B. S., T. Liu, et al. (2001). "Increased levels of oxidative stress markers detected in the brains of mice devoid of prion protein." J Neurochem **76**(2): 565-572.

Wuthrich, K. and R. Riek (2001). "Three-dimensional structures of prion proteins." Adv Protein Chem **57**: 55-82.

Yin, X. M., Z. N. Oltvai, et al. (1994). "BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax." Nature **369**(6478): 321-323.

Zahn, R., A. Liu, et al. (2000). "NMR solution structure of the human prion protein." Proc Natl Acad Sci U S A **97**(1): 145-150.