DEVELOPMENT AND CHARACTERIZATION OF NOVEL MONOCLONAL ANTIBODIES FOR STUDYING PRION PATHOGENESIS

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ABSTRACT OF THESIS

Chu-Chun Weng

The Graduate School
University of Kentucky
2011
DEVELOPMENT AND CHARACTERIZATION OF NOVEL MONOCLONAL ANTIBODIES FOR STUDYING PRION PATHOGENESIS

ABSTRACT OF THESIS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the College of Medicine at the University of Kentucky

By
Chu-Chun Weng

Lexington, Kentucky

Director: Dr. Glenn Telling,
Professor of Microbiology, Immunology and Molecular Genetics

Lexington, Kentucky

2011

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ABSTRACT OF THESIS

DEVELOPMENT AND CHARACTERIZATION OF NOVEL MONOCLONAL ANTIBODIES FOR STUDYING PRION PATHOGENESIS

Monoclonal antibodies (mAbs) recognizing different regions of PrP are potential tools in the study of prion diseases and immunotherapy. We used shuffled recombinant prion protein containing elk and mouse PrP as antigen to produce monoclonal antibodies in mice. We found that mAb 5C6 mapped to a discontinuous epitope comprised of amino acid 132 and 158 (mouse numbering). Monoclonal antibody 9E9 which maps to a unique N-terminal epitope at amino acid preferentially recognized cervid PrP. In contrast, the epitope of mAb 9H9 is located in the C-terminus and only reacted with mouse and hamster. The epitope for mAb 7H11 appears to be affected by the glycosylation of PrP and by the presence or absence of the disulfide bond. To confirm the epitopes of these mAbs, we constructed elk and mouse mutants both with and without reactivity to 5C6 and 9E9. We then used these mutants to investigate the effect of each epitope on the conversion of PrP\(^{C}\) to PrP\(^{sc}\). In one approach to map the epitopes of newly-generated monoclonal antibodies (mAbs), we generated a series of contiguous ten amino acids deletion constructs spanning amino acids 107 to 230 and expressed these recombinant proteins in mammalian cells (RK13) or bacteria. Using Western blotting, all deletion constructs could be recognized with antibodies to the extreme C-terminus of PrP, or the N-terminal region upstream of the structured globular domain of PrP. However, mAb 5C6 failed to react with all internally deleted PrP constructs expressed in mammalian cells, and to a lesser
extent bacterially produced mutant recombinant proteins. We confirmed the surprising result using the well-defined antibodies 6H4 and D18, which recognize epitopes in the same internal region as 5C6. Our results suggest the formation of an ultra-stable, SDS-resistant conformation in PrP harboring deletions mutations in the globular domain of PrP. We hypothesize that epitope burying within this stable conformation(s) precludes mAb recognition by 5C6, 6H4 and D18. It will be of extreme interest to determine the relationship of this previously undefined PrP conformation to the pathogenic process of PrP conformational change.

KEYWORDS: Prion, epitope, Monoclonal antibody
DEVELOPMENT AND CHARACTERIZATION OF NOVEL MONOCLONAL ANTIBODIES FOR STUDYING PRION PATHOGENESIS

By

Chu-Chun Weng

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Director of Thesis

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Director of Graduate Studies
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This work is dedicated to my family and my mentor, Dr. Glenn Telling. His support, understanding and encouragement have been exceptional.
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The project has been both challenging and rewarding. Many people have provided me with assistance and support for which I am grateful. I first would like to express my sincere gratitude to Dr. Glenn Telling, as he mentored me throughout this project. He is a true professional in every sense of the word. With his insightful scientific opinions, I have gained new perspectives and confidence in my research project.

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<td>BSE</td>
<td>Bovine Spongiform Encephalopathy</td>
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<td>CFA</td>
<td>Freund's complete adjuvant (CFA)</td>
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<td>CJD</td>
<td>Creutzfeldt Jacob disease</td>
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<tr>
<td>CWD</td>
<td>Chronic Wasting Disease</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked Immunosorbent Assay</td>
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<td>EK</td>
<td>Enterokinase</td>
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<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
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<tr>
<td>FFI</td>
<td>Fatal Familial Insomnia</td>
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<td>GPI</td>
<td>Glycosyl Phosphatidyl Inositol</td>
</tr>
<tr>
<td>GSS</td>
<td>Gerstmann-Straussler-Scheinker disease</td>
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<tr>
<td>MAb</td>
<td>Monoclonal Antibody</td>
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<td>MoPrP</td>
<td>Mouse PrP</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>PK</td>
<td>Proteinase K</td>
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<tr>
<td>PMSF</td>
<td>Phenyl Methyl Sulfonyl Fluoride</td>
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<td>PNGaseF</td>
<td>N-glycosidase F</td>
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<td>PrP</td>
<td>prion protein</td>
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<tr>
<td>PrP&lt;sup&gt;C&lt;/sup&gt;</td>
<td>Cellular isoform of the prion protein</td>
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<tr>
<td>PrP&lt;sup&gt;Sc&lt;/sup&gt;</td>
<td>Disease-associated isoform of the prion protein</td>
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<td>RK13</td>
<td>Rabbit epithelial kidney Cells</td>
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<td>RML</td>
<td>Rocky Mountain Lab strain of mouse-adapted scrapie</td>
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<td>TME</td>
<td>Transmissible Mink Encephalopathy</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>TSE</td>
<td>Transmissible Spongiform Encephalopathy</td>
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<tr>
<td>vCJD</td>
<td>variant Creutzfeldt Jacob disease</td>
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Chapter 1

Introduction

The Prion diseases

Prion diseases, also called transmissible spongiform encephalopathies (TSEs), are a group of fatal neurodegenerative diseases occurring in humans and animals. They are characterized by the development of spongiform degeneration associated with neuronal loss, reactive astrocytic gliosis, and occasionally amyloid deposition in the brains of infected hosts. The cause of prion diseases is an unconventional proteinaceous pathogen termed prion (Prusiner 1982). Unlike other infectious agents, the prion is resistant to many physical and chemical treatments such as Ultraviolet irradiation, heat or nucleases that are usually used to inactivate conventional pathogens. After such treatments, the infectivity of prions is not reduced (Alper, Cramp et al. 1967; Gibbs, Gajdusek et al. 1978; Prusiner 1982). Currently, no therapies or cures are available.

Prion diseases of humans are unique in that they can be inherited in an autosomal dominant manner, occur sporadically, or result from infection. The human diseases include Creutzfeldt-Jacob disease (CJD), kuru, fatal familial insomnia (FFI), Gerstmann-Straussler-Scheinker disease (GSS), and variant CJD (vCJD). Approximately 85% of human prion disease are sporadic CJD which occurs at a frequency of one to two cases per million people per year, usually between the age of fifty to sixty years (Wadsworth J.D.F. 2007). Kuru was the
first recognized that transmissible human prion disease. Kuru occurs in the forelinguistic group of eastern the highlands province of Papua New Guinea where they have ritualistic cannibalism (Gajdusek, Gibbs et al. 1967). Children and women, who were the main consumers of elder tribe members’ brains, were most often affected by kuru (Alpers and Gajdusek 1965). Several familial human prion diseases exist including FFI and GSS. These diseases account for 10% to 20% of all human prion diseases. The age of clinical onset in familial prion diseases is generally earlier, and the incubation time is longer than in sporadic prion diseases (Collinge, Whitfield et al. 2008). In the 1990s, several cases of CJD, termed variant Cretuzfeld-Jakob disease (vCJD), were recognized in young adults, generally in their early twenties, with a clinical presentation in which behavioral and psychiatric disturbances predominated. Biochemical and neuropathological data indicated a possible association with bovine spongiform encephalopathy (BSE) in cattle (Bruce, Will et al. 1997; Hill, Desbruslais et al. 1997). To date with approximately 200 individuals have developed disease, most of the cases were occurred in Great Britain. This unique feature has allowed retrospective studies designed to determine the true frequency of disease, which suggests than many more individuals are incubating the disease than have currently developed clinical symptoms worldwide (Ironside, Hilton et al. 2000). At present, there is a concern that some individuals exposed to BSE might be asymptomatic carriers of infectivity (Brandel, Delasnerie-Laupretre et al. 2000) and that these people might pose a risk of further transmission of the infection to others (e.g., blood transfusions, donors for corneal transplantations)
In animals, prion diseases can either occur naturally or as the result of consumption of contaminated food and are more common than in humans. In 1986, a new prion disease occurring in cattle of United Kingdom was termed Bovine spongiform encephalopathy (BSE). BSE is amplified through the bovine food chain via eating feed containing rendered meat and bone meal contaminated with bovine brain and spinal cord from infected cattle (Wilesmith, Ryan et al. 1991). This disease has affected cattle worldwide after the first case in United Kingdom. In 1996, a variant CJD has been reported in humans (Will, Ironside et al. 1996). Extensive biochemical and pathogenetic evidence indicated that vCJD is caused by consumption of with BSE-contaminated food.

Chronic Wasting Disease (CWD) which was first discovered at a research facility in north central Colorado in 1967 is a TSE affecting mule-deer (Williams and Young 1980), Rocky Mountain elk (Williams and Young 1982), white-tailed deer (Spraker, Miller et al. 1997), black-tailed deer (Williams and Young 1980), and moose (Baeten 2007). This disease was identified as a TSE by identification of neuronal vacuoles (Williams and Young 1980) and deposition of plaques or aggregated prion protein in the brain (Spraker, Zink et al. 2002). These animals contained prion infectivity in the brain (Williams and Young 1992; Browning, Mason et al. 2004) and in skeletal muscle (Angers, Browning et al. 2006). CWD has rapidly spread to several states including New York and West Virginia from a small region in southeastern Wyoming, northeastern Colorado, southwestern Nebraska, and a small part of Canada (Sigurdson 2007). It has also been found in imported elk in South Korea (Sohn, Kim et al. 2002). The distribution of the
disease in focal spots suggests the possibility that outbreaks may have occurred as a result of spillover from infected game farms rather than spread as a result of the natural movement of free-ranging animals (Sigurdson 2007). CWD is the only prion disease of wild animals that has been documented to be transmitted horizontally. The source and route of transmission are currently unknown (Miller, Wild et al. 1998; Miller, Williams et al. 2000). Additionally, CWD is highly contagious between cervids. However its ability to transmit to other species is not entirely clear, especially to those that share lands with CWD-affected cervids or to humans consuming the meat of CWD-affected animals.

Scrapie is a natural prion disease of sheep and has existed over 200 years with no evidence suggesting transmission to humans despite the existence of several well-characterized strains (Bruce 1993). There are multiple environmental sources of infection to transmit between sheep or flocks. Transmissible Mink Encephalopathy (TME) is a prion disease of mink that has been found sporadically in farmed mink in several countries. It was first recognized in 1947 in Wisconsin and Minnesota (Eckroade 1973). TME has been experimentally transmitted to several species including cattle (Hamir 2006), sheep and goats (Hadlow 1987), monkeys (Eckroade 1970), skunks and ferrets (Eckroade 1973), and raccoons (Eckroade 1973; Hamir, Miller et al. 2004). The most interesting transmission of TME is from an affected mink in Wisconsin to hamsters. After serial passage, it presents with two distinct clinical profiles termed hyper (HY) and drowsy (DY) (Bessen and Marsh 1992). These two strains of TME were found with different transmission profiles and biochemical properties in hamsters.
Molecular biology of prion proteins (PrP<sub>C</sub> and PrP<sub>Sc</sub>)

The normal form PrP, cellular prion protein (PrP<sub>C</sub>), is a molecule ubiquitously expressed in mammalian cells. It is synthesized in the secretory pathway on endoplasmic reticulum (ER)-attached ribosomes as a 253 amino acid protein before post-translational modification. Mature PrP<sub>C</sub> is found as a glycosyl phosphatidyl inositol (GPI)–linked glycoprotein with a disulfide bond which is enriched in cell membrane and is highly conserved in mammals. The N-terminal region of this molecule is unstructured, but it still contains two defined and conserved regions. The first region consists of five repeats amino acid sequence called octa-repeat region (Riek, Hornemann et al. 1997; Smith, Drake et al. 1997). This region has been proposed to be important in copper binding and might be involved in prion pathogenesis (Brockes 1999). The second region contains a highly hydrophobic profile, which was originally termed transmembrane region 1 (Hay, Barry et al. 1987; Shyng, Moulder et al. 1995). The function of this domain is still unclear, but it is relevant to prion pathology and necessary for PrP<sub>C</sub> α-cleavage (Lutz, Brabeck et al. 2010). The carboxyl terminus of PrP<sub>C</sub> contains of three α-helices, and two anti-parallel β-sheets. There are two asparagine residues that for glycosylation and two cysteine residues will form a disulfide bond links α-helices two and three (Riek, Hornemann et al. 1996; Prusiner 1998; Harris 2003). The pathogenic form PrP (PrP<sub>Sc</sub>) is derived from PrP<sub>C</sub> and has identical amino acid sequence with PrP<sub>C</sub> (Oesch et al., 1985; Chesebro et al., 1985; Stahl et al., 1993).
PrP is a high α-helical content structure containing 42% α-helix and 3% β-sheet. When PrPSc is formed, some of the α-helix is converted into β-sheet. PrPSc is a high β-sheet construct contains 43% β-sheet increasing from 3% (Caughey, Dong et al. 1991; Pan, Baldwin et al. 1993; Pergami, Jaffe et al. 1996). Because of this conformational transition, PrPSc has lower solubility in non-denaturating detergents, a high tendency to aggregate and partial resistance to proteinase K (PK). Unlike PrPrSc, PrPC is PK sensitive and detergent soluble (Table 1). Because of these properties, the protease resistance of PrP is a way to distinguish the two forms of PrP (Oesch, Westaway et al. 1985). After treatment with PK, PrPSc is seen as three bands: the di-glycosylated, mono-glycosylated, and un-glycosylated form in western blot (Bolton, McKinley et al. 1982; Oesch, Westaway et al. 1985), while PrPC is degraded.

**Modification and trafficking in the cell**

The processing of mature PrPC includes the cleavage of the N-terminal signal sequence by a signal peptidase in the ER, removal of the C-terminal signal peptide and replacement with a glycosylphosphatidylinositol (GPI) anchor, formation of a disulfide bond between two cysteine residues, and glycosylation at two asparagines (Prusiner 1998; Harris 2003). PrPC is then delivered to the cell surface and anchored to the outer leaflet of neurons and other cell types in lipid rafts via the GPI anchor (Figure 1). PrPC is also recycled between the plasma membrane and endocytic compartment (Shyng, Huber et al. 1993). PrPC is highly expressed on the surface of neurons and lymphocytes, but is also
expressed on dendritic cells and cells of the monocyte lineage (Kitamoto, Muramoto et al. 1991). There are two other topological forms of PrP\(^C\) that are both in the transmembrane. One is found with the N-terminus in the lumen of the ER and is called Ntm-PrP and the other is in the opposite orientation and called Ctm-PrP (Hegde, Mastrianni et al. 1998). Mature PrP\(^C\) has a molecular weight of approximately 33-35kD containing an N-terminal unstructured flexible region with an octapeptide repeat that can bind copper and a C-terminal structured region with three alpha helices and two short beta sheets (Figure 1.1).

Several studies indicate that the conversion of cellular PrP\(^C\) to a protease resistant PrP\(^Sc\) is a posttranslational event that occurs after the protein reaches the lipid raft on the cell surface. (Caughey and Raymond 1991; Borchelt, Taraboulos et al. 1992; Taraboulos, Raeber et al. 1992). The reason why PrP\(^C\) has to reach the membrane before it converted to PrP\(^Sc\) is still unknown. One possibility might be the posttranslational modifications for PrP\(^C\) are necessary for its conversion after reaching the plasma membrane. One possibility is PrP\(^C\) functions as a receptor that mediates PrP\(^Sc\) internalization during infection. Alternatively the lipid and protein environment at the plasma membrane might be favorable for the PrP\(^C\) and PrP\(^Sc\) interaction and conversion, or a factor needed for the transconformation could be localized specifically at the plasma membrane (Prusiner 1998; Campana, Sarnataro et al. 2005).

When PrP\(^C\) was transported to the cell surface, PrP\(^C\) undergoes an endoproteolytic cleavage event The C1 cleavage site (Chen, Teplow et al. 1995)
is located between 109/110 in mouse sequencing numbering, producing a 17-KDa C-terminal fragment. When PrP\textsuperscript{C} is converted to PrP\textsuperscript{Sc}, the conformational change of PrP makes the C1 cleavage site instead of a new cleavage site termed C2 cleavage producing a 21-Kda C-terminal fragment. The C2 cleavage site is between 88 and 89 in mouse numbering.

**Function of PrP**

PrP\textsuperscript{C} has been found in several mammals, and is highly conserved (Harris, Lele et al. 1993; Windl, Dempster et al. 1995). The expression of PrP\textsuperscript{C} can be detected in most tissues except in kidney and liver, and is particularly high expressed in the central nervous system (Miele, Alejo Blanco et al. 2003). PrP\textsuperscript{C} is ubiquitously expressed, but its function still remains unknown. Several groups have generated PrP knock out (Prnp\textsuperscript{-/-}) mice in an effort to study the function of PrPC. In 1992, Büeler group in Zurich deleted the open reading frame of Prnp gene to create the Zurich I Prnp\textsuperscript{-/-} mouse. The resulting mice were devoid of PrP\textsuperscript{C} and found to be developmentally normal (Büeler et al., 1992). However, these mice do display demyelination in the peripheral nervous system with age, but without the development of clinical signs (Nishida et al., 1999). In 1994, another knock out mouse model of PrnP\textsuperscript{-/-} (Edinburgh) was generated and also developed normally (Manson, Clarke et al. 1994). Later, there was another Prnp\textsuperscript{-/-} mice developed by Sakaguchi group known as Prnp\textsuperscript{-/-} (Nagasaki). This knockout mouse strain involved deleting the open reading frame of PrnP gene and ~1Kb of the intron 5’ to the open reading frame including the splice acceptor site (Moore et al., 1999; Sakaguchi et al., 1996). This The deletion in the non coding region caused
unusual intergenic splicing between the remaining PrP gene and a Prnd gene encoding a protein called Doppel (Dpl), which is 16 kb downstream of the Prnp locus (Moore, Lee et al. 1999). Mice did develop ataxia and loss of cerebellar Purkinje cells with age (Sakaguchi, Katamine et al. 1996) but this was attributed to the overexpression of Doppel, not the absence of PrP^C (Moore et al., 1999; Sakaguchi et al., 1996). The doppel protein shares 24% homology in amino acid sequence with PrP, is primarily expressed in the testis, and is absent in the CNS of wild type mice but present in the CNS of Nagasaki Prnp^/- mice (Silverman, Qin et al. 2000). Several studies demonstrated PrPC is an important factor for developing Prion disease by examining the susceptibility of Zurich PrnP^/- mice to scrapie (Büeler, Aguzzi et al. 1993). However, PrP knockout mice are resistant to prion disease after inoculation with prions (Büeler, Aguzzi et al. 1993; Prusiner, Groth et al. 1993). But susceptibility can be restored after introducing PrP^C into these mice (Büeler, Aguzzi et al. 1993). These studies provide further evidence that PrP^C is required for the conversion process and formation of PrP^Sc.

**Species Barrier**

The species barrier or transmission barrier was thought to be caused by differences in the primary sequence of host and donor PrP (Harris 1999; Horiuchi, Priola et al. 2000). It refers to the restriction of transmission of prion diseases between different species. It depends on the species being infected and the specific strain of TSE agent involved. For example, mouse scrapie can be transmitted to Syrian hamsters with a long incubation time (over 370 days) but transmitted to mice with shorter incubation time (around 100 days) (Kimberlin and
Walker 1978). The 263K hamster scrapie strain transmitted into Syrian hamsters has 60-day incubation time, but there is no clinical disease observed when it is transmitted to mice (Kimberlin, Walker et al. 1989). Mouse PrP and Syrian hamster PrP differ at only 12 of 209 amino acids. There are many factors that influence the species transmission barrier. Understanding its molecular basis and mechanism is valuable after the discovery that vCJD is the result of a transmission of BSE from cattle to human (Collinge, Sidle et al. 1996; Hill, Desbruslais et al. 1997). It raises concerns about the possibility of transmission of prions from other species to humans, for example CWD from deer and elk. The differences in primary sequence of PrP and prion strain are two factors that contribute to the species transmission barrier.

**Prion Strains and Polymorphisms**

Prion strains are characterized by difference of incubation time, distribution of pathological lesions that they produce in the central nervous system of recipient animals and clinical symptoms. (Telling 2004; Angers, Kang et al. 2010). The influence of genetic variability on susceptibility to disease and pathogenesis has been reported. Susceptibility or resistance to scrapie in sheep is associated with polymorphisms at codons 136 (A/V), 154 (R/H) and 171 (Q/R/H) of the sheep Prnp gene (Elsen, Amigues et al. 1999). Scrapie susceptibility is conferred by the VRQ and resistance by ARR and AHQ polymorphisms. ARQ and ARH are with an intermediate situation. The polymorphisms in all species of cervids are also currently investigated (Schatzl, Wopfner et al. 1997; O'Rourke, Baszler et al. 1998; Johnson, Johnson et al. 2003; O'Rourke, Spraker et al. 2004; Jewell,
Conner et al. 2005; Johnson, Johnson et al. 2006; Green, Browning et al. 2007; Meade-White, Race et al. 2007). One polymorphism found in elk is codon 132 encoding either methionine or leucine (Schatzl, Wopfner et al. 1997; O'Rourke, Baszler et al. 1998). This polymorphism is important because this residue corresponds to codon 129 in the human prion protein that has been shown the susceptibility to vCJD (Collinge, Sidle et al. 1996).

Immunological approach to study prion diseases

Several anti-PrP antibodies have been produced to detect prions and diagnose prion diseases via immunoblotting. The similarity in the primary sequence of donor PrPSc in comparison to PrP\textsuperscript{C} of the recipient species seems to play a crucial role in the species barrier. The monoclonal antibody 3F4 recognized amino acids from position 109 to 112 in human PrP (Barry and Prusiner 1986; Kascsak, Rubenstein et al. 1987) and has been one of the most commonly used tools in studying prion disease. It reacts strongly to human and hamster PrP and requires two methionine residues at position 109 and 112 in human PrP. PrP from other species which lack one or both methionine residues does not react with 3F4. Some groups introduced 3F4 epitope into MoPrP by replacing leucine 108 and valine 111 with methionines present in human and hamster PrP and have demonstrated the 3F4 required these two methionines for recognition. However, because the epitope of 3F4 is close to the endoproteolytic processing site of PrP, the introduction of this tag partially interfered with the conversion process and substantially reduced the total consistent PrPSc accumulation in infected cells (Priola, Caughey et al. 1994; Lund, Olsen et al. 2007). MAb L42 is another
antibody that reacts with PrP\(^C\) in a broad range of species including sheep, cattle, goat, pig, human, dog, cat, mink, rabbit, and guinea pig but not mouse and hamster in immunoblotting analysis (Vorberg, Pfaff et al. 2000). MAb L42 was raised by immunization of mice with an oligopeptide covering the ovine PrP\(^C\) sequence 145-163 (corresponding to position 141-159 of murine PrP). Some groups were using antibody-based immunotherapy against prion disease. However, the difficulty of using antibody to cure prion disease in humans still needs to overcome is host tolerance to PrP\(^C\) (White, Enever et al. 2003).
Thesis research

Monoclonal antibodies (mAbs) are indispensable in studying and diagnosing prion diseases. It is also useful for immunotherapy. The goal of this project is to produce and characterize novel monoclonal antibodies against PrP. To achieve the goal we used PCR-based method to shuffle and generate a novel PrP structure containing mouse PrP and cervid PrP. We will be utilizing these novel anti-PrP monoclonal antibodies to study the species barrier, conversion mechanism of PrP\(^C\) to PrP\(^S\), and the secondary structure conformation.

The results section includes how we made a shuffled recombinant prion protein composed of mouse and elk PrP. We used this mixed recombinant prion protein as antigen to produce novel monoclonal antibodies in PrP-null mice. We used the feature of conserved PrP sequence of variant species to screen our hybridoma cells, we identified four monoclonal antibodies. We found that mAb 5C6 mapped to a discontinuous epitope comprised of amino acid 132 and 158 (mouse numbering). Monoclonal anibody 9E9 which maps to a unique N-terminal epitope at amino acid preferentially recognized cervid PrP. In contrast, the epitope of mAb 9H9 is located in the C-terminus and only reacted with mouse and hamster. The epitope for mAb 7H11 appears to be affected by the glycosylation of PrP and by the presence or absence of the disulfide bond. In result section 2, in order to confirm the epitopes of these mAbs, we constructed elk and mouse mutants both with and without reactivity to 5C6 and 9E9. We then used these mutants to investigate the effect of each epitope on the conversion of PrP\(^C\) to PrP\(^S\). The results may provide new insights into the biology of PrP\(^C\) and PrP\(^S\).
In result section 3, it provides another approach to map monoclonal antibodies. We generated a series of contiguous ten amino acids deletion constructs spanning amino acids 107 to 230 and expressed these recombinant proteins in mammalian cells or bacteria. Using Western blotting, all deletion constructs could be recognized with antibodies to the extreme C-terminus of PrP, or the N-terminal region upstream of the structured globular domain of PrP. However, mAb 5C6 failed to react with all internally deleted PrP constructs expressed in mammalian cells, and to a lesser extent bacterially produced mutant recombinant proteins. We confirmed the result using the well-defined antibodies 6H4 and D18, which recognize epitopes in the same internal region as 5C6. Our results suggest the formation of an ultra-stable, SDS-resistant conformation in PrP harboring deletion mutations in the globular domain of PrP. We hypothesize that epitope burying within this stable conformation(s) precludes mAb recognition by 5C6, 6H4 and D18. It will be of extreme interest to determine the relationship of this previously undefined PrP conformation to the pathogenic process of PrP conformational change.
Figure 1.1 Schematic diagrams of mouse PrP primary structure and its posttranslational modifications. The N-terminal region of PrP is unstructured and the C-terminal region is structured. In the extreme N terminus is a secretory signal peptide (orange). Region 51-90 consists of 5 octarepeats, which was suggested to play a role in copper binding (Brockes 1999). The royal blue region is the structured region containing 3 α-helices denoted α1, α2, α3 in red and two β-sheets denoted β1, β2 in red. N-linked glycosylation site (Asn180, Asn196) is indicated by two red hexagons. Cysteines 178 and 213 form a disulfide bridge. Glycosyl phosphatidyl inositol (GPI) anchor signal peptide is located at the end of C-terminus and labeled green. There are two endoproteolytic cleavage sites, C1 and C2, indicated by arrows. C1 cleavage site is located at position His110/Val111 and will produce a ~17kDa C1 fragment. C2 cleavage site is located at position Trp88/Gln89 and will produce a ~21kDa C2 fragment.
Table 1. Comparison of PrP properties

<table>
<thead>
<tr>
<th>PrP&lt;sup&gt;C&lt;/sup&gt;</th>
<th>PrP&lt;sup&gt;Sc&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble</td>
<td>Insoluble</td>
</tr>
<tr>
<td>α-helix rich</td>
<td>β-sheet rich</td>
</tr>
<tr>
<td>Proteinase- sensitive</td>
<td>Proteinase-resistance</td>
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<tr>
<td>monomers</td>
<td>aggregates</td>
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Chapter 2
Materials and methods

Preparation of antigen using DNA shuffling

PCR amplification of elk and mouse PrP coding sequences: 100 ng of recombinant plasmids (pCAGGS-MoPrP or pCAGGS-elkPrP) carrying elk or mouse prion protein gene were amplified with pfu polymerase (Stratagene) as following condition: pre-denaturation 95°C/3min; 95°C /40sec; 60°C /40sec;72°C /60sec. PCR products were purified by running 1.2% agarose followed by column purification (Premega). The concentration of purified PCR products was quantified by running 1.2% agarose gel with DNA standard.

DNase I digestion of mixed MoPrP and elkPrP DNA fragments: 3 μg of mixed PCR products of MoPrP and ElkPrP (1:1) was incubated at 15°C for 10 min, then add 0.15 unit of DNase I(Roche), incubated at 15°C for 10min, the reaction was terminated by adding 10μL of stop solution and inactivated DNase I at 96°C for 10min. The DNase I digested DNA was separated with 2% low-temperature agarose gel (Invitrogen) along with 10-bp DNA ladder. DNA fragments with the length from 10 to 300bp were purified with phenol/chloroform method.

Re-assembly of small DNA fragments: 30 μL of DNase I digested DNA fragments, 5 μL of Pfu Ultra buffer, 1μL of dNTP (Roche), 1μL of Pfu Ultra were mixed and the volume was adjusted to 50μL with ddH2O. The re-assembly PCR were performed for 45 cycles as the following conditions: 94°C/3min; 94°C/30sec; (27°C +1°C/cycle) /1min; 72°C/(1min+4sec/cycle); the PCR product was maintained at 72°C for 10min and then stored at 4°C.
PCR amplification of reassembled PCR products: The reassembled DNA fragments were amplified with primers with the addition of EcoRV and BamHI at 5’ends of upstream or downstream primers for 35 cycles as the following conditions: pre-denaturation 94°C/3min; 94°C/45sec; 47°C with increase 0.7°C/cycle 1min; 72°C/1min. PCR products were kept at 72°C for 10min. The shuffled DNA fragments were digested, purified and then inserted into pIRESpuro3 vector.

PCR-RFLP prescreening the chimeric DNA: Bacterial Colony-PCR was performed and PCR products were purified with Wizard DNA purification Kit (Promega) and digested with StuI and AluI.

DNA sequencing: plasmids were extracted according to QIA miniprep protocol (Qiagen), and sequenced with CEQ8000 instrument.

Cell cultures
Rabbit epithelial kidney RK13 cells (ATCC, Manassas, VA) were maintained in 5% CO₂ at 37 °C in DMEM with 10% FBS.

Constructs
The elk PrP or mouse PrP coding sequence was amplified by polymerase chain reaction (PCR) with primers containing AflII and EcoRI restriction endonuclease recognition sites at the 5’ and 3’ ends respectively. Digested amplicons were inserted into AflII- and EcoRI-cleaved pIRESpuro3 vector (Clontech, Mountain View, CA). Following confirmation of the elk PrP coding sequence in the recombinant vector, RK13 cells were transfected with pIRESpuro3 containing elk PrP, mouse PrP or empty vector to produce RKE, RKM and RKV cells respectively.
**Generation of transfected cells**

Cells were plated in 6-well plates one day prior to transfection. Transfection mixtures were prepared by mixing 2 μg of plasmid and 6.3 μl of lipofectamine 2000 (Invitrogen, Carlsbad, CA) in Opti-MEM medium (Invitrogen, Carlsbad, CA). Prior to addition of 200 μl transfection mixture, cells were rinsed with Opti-MEM. Following addition of transfection mixtures, 0.8 ml of complete medium containing 10 % FBS was added to cell monolayers. Following passage to 10 cm plates 24 hr later, transfected cells were grown in complete medium containing 1 μg/ml puromycin. Selection medium was changed every 3 days.

**Infection with brain homogenate in Cell culture**

Cells (2 x 10⁵) were split to 6 well plates one day before prion infection. 10 % brain homogenates diluted in Opti-MEM medium to 0.2 - 2% were added to cell monolayers, in a volume of 1 ml per well. After 5 h, 2 ml of Opti-MEM medium containing 15 % FCS was added. Cells were split into 10 cm plates, followed by 2 more passages. Cell lysates were harvested at passage 3 for Western blotting.

**Immunoblotting**

**RK13 Cell lysate:**

Cultured cell were treated with cold lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 0.5% Igepal CA-630) and total protein concentration was determined by bicinchoninic acid (BCA) assay (Pierce Biotechnology Inc., Rockford, IL). Lysates were either untreated or treated with 40 mg/ml proteinase K (PK) for one hour at 50°C and the reaction was terminated with 4 mM phenyl methyl sulfonyl fluoride (PMSF) in final concentration. PrP<sup>Sc</sup> in cell culture lysates was purified by centrifugation for 1 h at 100,000 x g at 4°C. Samples were boiled
in the presence of SDS for 10 min prior to electrophoresis. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% Tris-glycine gels, and electrophoretically transferred to PVDF-FL membranes (Millipore, Billerica, MA). Membranes were blocked in Tris-buffered saline with 0.05% Tween-20 and 5% non-fat milk, incubated with anti-PrP monoclonal antibody (mAb) 6H4 (Prionics AG, Schlieren-Zurich), or other in-house mAb followed by HRP-conjugated sheep anti mouse IgG secondary antibody. Membranes were developed using ECL-plus detection (Amersham), and analyzed by using a FLA-5000 scanner (Fuji).

**Brain homogenate:**

Animals whose death was obviously imminent were euthanized by CO₂ inhalation and cervical dislocation. Brain tissues from sacrificed mice were isolated rapidly on dry ice and stored frozen at -80°C. Ten % brain homogenates in phosphate buffered saline (PBS) lacking calcium and magnesium ions were prepared on ice by repeated passage through 18-, followed by 22-gauge needles. 10 % brain homogenates were aliquoted and stored in -80°C. Total protein concentration was determined by bicinchoninic acid (BCA) assay (Pierce Biotechnology Inc., Rockford, IL). Equivalent amounts of total protein were boiled at 100°C in an equal volume of 4X sodium dodecyl sulfate (SDS) buffer (100 mM Tris HCl, 4% SDS, 0.2 % bromophenol blue, 20 % glycerol) for 10 minutes. Boiled samples were then electrophoretically separated on a 12 % SDS-polyacrylamide gel (acrylamide, SDS, ammonium persulfate and TEMED - Bio Rad). PK digestion of brain homogenates were performed with the addition 2% sarkosyl and incubated for 1 hr at 37 °C at a PK concentration of 40 µg/ml. Digestion was terminated by
the addition of phenyl methyl sulfonyl fluoride (PMSF) to a final concentration of 4 mM. Deglycosylation of PrP was achieved by digestion with recombinant PNGase F for 1 hour at 37°C, as specified by the supplier (New England Biolabs, Beverly, MA). Following the addition of 4x non-reducing SDS loading buffer, samples were boiled for 10 minutes at 100 °C. Proteins were resolved by 12% SDS-PAGE and electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes for later development PVDF-FL membranes (Millipore) for fluorescent scanning. Following electrophoretic transfer, membranes were incubated for 45 min in 5% (w/v) low fat milk in Tris buffered saline containing Tween 20 (TBST) to block non-specific protein binding. Membranes were probed two hours at 4 °C with monoclonal antibody and incubated 1 hour at room temperature with horse raddish peroxidase (HRP)-conjugated goat anti-Mouse secondary antibody. Blots were developed using ECL-Plus detection (Amersham Biosciences, Piscataway, NJ) and analyzed by using a FLA-5000 scanner (Fuji).
Chapter 3
Results

Section 1: Production and Characterization of monoclonal antibodies

A. Generating immunogen containing murine and cervid PrP using DNA shuffling

Many anti-PrP antibodies have been produced. To create anti-PrP monoclonal antibodies with novel epitopes, we generated new PrP sequences by reassembling the sequences of two different species in vitro. The similarity between mouse and elk PRNP coding sequence is 81.54% and protein similarity is 85.56% (Figure 2.1). Dr. Jifeng Bian, a scientist in our laboratory, used polymerase chain reaction (PCR) to amplify mouse and elk PRNP coding sequences. He digested the PCR products with DNase I to generate a random pool of fragments. These fragments were reassembled by repeated cycles of annealing in the presence of DNA polymerase. Some of these fragments were overlapping. Recombination can occur when a fragment from one species becomes the template for a fragment from the other, and vice versa, causing template switch (Stemmer 1994; Stemmer 1994). With the resulting chimeric PrP constructs, we were able to produce a panel of antibodies which can recognize mouse PrP, elk PrP or both. Roughly one hundred sequences containing mouse and elk PrP were produced. There are 30 amino acids which differ between mouse and elk PrP sequence. Three constructs, shuffled PrP #3, #34 and #68, with equal distribution of mouse and elk PrP sequence, were chosen for immunization. In Figure 2.1, red squares represent mouse PrP
sequence and green squares represent elk PrP sequence. The middle sequence is shuffled PrP #68, with mouse PrP sequence above and elk PrP sequence below for reference. Of the 30 amino acids which differ between mouse and elk, this chimera share 14 with mouse PrP and 16 with elk PrP.

In order to generate recombinant shuffled protein, Dr. Hae-Eun Kang, a postdoc in our laboratory, cloned these three shuffled PrPs into pET-100/D-TOPO vector (Invitrogen) containing polyhistidine site and enterokinase (EK) recognition site in the N-terminus. She utilized the polyhistidine site to purify recombinant protein with Ni-NTA resin. After purification, she used EK, which cleaves at the C-terminus of the EK recognition site, to remove N-terminal tags without leaving extraneous amino acid residues on the shuffled protein (Figure 2.2).

**B. Screening of hybridoma cells**

Fusing myeloma cells with spleen cells from a mouse that has been immunized with the desired antigen is a popular method to produce monoclonal antibodies (Kohler and Milstein 1975). In our study, we reassembled PrP sequences and chose three constructs containing an equal percentage of the elk and mouse PrP sequences. We expressed these constructs in bacteria, purified them, and used them as immunogen. In order to achieve an immune response, PrP-null mice (Prnp−/−) were used (Bueler, Fischer et al. 1992). Each shuffled PrP was used to immunize two PrP-null mice. Freund's complete adjuvant (CFA) was used to emulsify the antigen. CFA is effective in stimulating immunity and may increase the production of immunoglobulins. After immunization, we collected blood and
detected the immune response via enzyme-linked immunosorbent assay (ELISA).
It was found that one mouse immunized with shuffled PrP #68 had an increased
immunogenic response to PrP (Figure 2.3) and spleen cells from this mouse were collected. After fusion of spleen cells with myeloma cells, we selected a series of hybridoma cells producing monoclonal antibodies recognizing recombinant mouse PrP (rMoPrP) or elk PrP (rElkPrP) in the ELISA. Screening of hybridomas revealed several antibodies that reacted strongly against recombinant PrP in the ELISA and western blot. Four of the antibodies that had strong reactivity were picked for further characterization. They are hybridoma cell lines 5C6, 9E9, 7H11 and 9H9.
Figure 2.1 Comparing primary structures of mouse PrP, elk PrP and shuffled PrP. This figure is to show the different sequencing result between mouse and elk PrP in primary structure and the distribution of these two species PrP in the shuffled PrP #68. The top is mouse PrP sequence and the bottom is elk PrP sequence. The middle sequence is our shuffled PrP #68. The difference between mouse and elk PrP is indicated by a red box (mouse) or a green box (elk). The amino acid sequence of shuffled PrP #68 was labeled as indicated. The numbering was designated in elk numbering. The locations of secondary structure are indicated by brown arrows (beta-sheet) and blue ribbons (alpha-helix).
Figure 2.2 SDS-PAGE analysis of purified shuffled PrP recombinant protein. ‘E’ is eluted PrP with His in urea buffer at pH4.5. ‘Su’ is recombinant PrP in NaOAc buffer at pH5.9 after EK treatment. ‘Pt’ is precipitated recombinant PrP in NaOAc buffer at pH4.5 after EK treatment. ‘C’ is control without EK treatment. The PAGE was stained (a) coomassie blue, or blotted with (b) mouse monoclonal antibody anti-PrP 6H4 with 1:10,000 dilution or (c) His-probe.
Figure 2.3 Titration of anti-PrP antibodies in the immunized mice. 96 well ELISA plate was coated with 100ul of 1µg/ml recombinant shuffled PrP solution per well. Blood serum was prepared from immunized mouse tails. Color development was done with ABST peroxidase solution, and the absorbance of samples was measured at 405 nm in ELISA plate reader. The x-axis is the dilution factor of the serum. Blue Square indicates the response of one immunized mouse with shuffled PrP#68. Red triangle represented second mouse immunized with shuffled PrP#68. Grey triangle represents mAb 6H4 as a control.
C. Characterization of anti-PrP monoclonal antibodies

To determine further specificity of the anti-PrP mAbs, western blot and immunohistochemical analyses were performed. Mammalian PrP (being 95% conserved) has a higher homology of amino acid sequences as compared to PrP in other species (Oesch, Westaway et al. 1985; Robakis, Sawh et al. 1986). To characterize these antibodies, we utilized transgenic (Tg) mouse brain homogenates, which were engineered to express PrPs from different species. Tg(MCerB+C) is a transgenic line containing mouse residues predicted to associate with hypothetical mouse protein X and cervid residues predicted to interact with PrP\(^{Sc}\) from cervids with CWD. All brain homogenates were prepared with 10% w/v sterile PBS lacking Ca\(^{2+}\) and Mg\(^{2+}\). The total protein content was measured by bicinchoninic acid assay (BCA). The non-diluted supernatant of the hybridoma cell culture was used as the first antibody. The previously characterized anti-PrP monoclonal antibody (MAb) 6H4 (Korth, Stierli et al. 1997) was used as a positive control. MAb 6H4 recognizes a single linear epitope, DYEDRYYRE, corresponding to positions 144–152 of human PrP. The mAb 6H4 reacted with 10% brain homogenates from a broad range of various Tg mice (Figure 2.4).
Figure 2.4 Western blot analysis of various Tg mouse brain homogenates with 6H4. All Transgenic mouse brain homogenates were prepared with 10% w/v sterile PBS lacking Ca\(^{2+}\) and Mg\(^{2+}\). Total protein content was measured by bicinchoninic acid assay (BCA). Each lane was loaded with 25 µg brain homogenate. KO is represented with Tg Prnp\(^{-/-}\) background mice, and is a negative control. Tg(MoPrP)4112 expresses high levels of mouse PrP in the knockout background. Tg(DeerPrP)1536 expresses deer PrP on the knockout background whereas Tg(ElkPrP)5037 expresses elk PrP, Tg(BoPrP)3705 expresses bovine, Tg(OvPrP-ARQ)3533 expresses one sheep allele of PrP, with arginine at residue 136 and Tg(OvPrP-VRQ)4166 expresses sheep PrP\(^{C}\) with valine instead. Tg(EqPrP)5525 expresses horse PrP\(^{c}\) on knockout background. Tg(HuPrP-M129)6816 expresses human PrP with methionine at residue 129 in knockout background mouse whereas Tg(HuPrP-V129)7826 expresses valine in residue 129. Tg(MCerB+C)849, is a chimeric line which expresses the mouse residues predicted to associate with mouse protein X and the cervid residues predicted to interact with PrP\(^{Sc}\) from cervids with CWD. Membrane was probed with mAb 6H4 in 1:10000 dilution, a comercial anti-PrP monoclonal antibody.
1) Monoclonal Antibody 5C6

To characterize the reactivity of 5C6 with PrP from different species, we prepared 10% brain homogenates from Tg mice expressing various PrP<sup>C</sup> sequences including mouse PrP, deer PrP, elk PrP, bovine PrP, sheep PrP, equine PrP, and human PrP. From the western blot analysis, mAb 5C6 did not react with sheep PrP<sub>VRQ</sub>, which has a polymorphism at residue 136 with sheep(ARQ) (Figure 2.5A). According to Figure 2.4, there is PrP expression in the brain homogenate of sheep (VRQ). Our collaborator, Dr. Jason Bartz, helped us to further characterize 5C6 by probing samples from his laboratory. He found that mAb 5C6 reacted with brain homogenates of a hamster, mink, ferret and cow, but did not react with a squirrel monkey brain homogenate (Figure 2.5B). Based on the western blot analysis with Tg mouse homogenates and other mammals’ brain homogenates, 5C6 can efficiently recognize mouse, elk, deer, cow, sheep (ARQ), human and horse PrP. Sheep (VRQ) and squirrel monkey have no reactivity with 5C6. According to these results and comparing the primary structure of various mammals, the epitope of mAb 5C6 requires alanine and asparagine in positions 132 and 158 of mouse PrP, respectively. (Figure 2.6)
Figure 2.5 Western blot analysis to assess 5C6 epitope via brain homogenates of (A) Tg mice and (B) other mammals. (A) 25 μg transgenic mouse brain homogenate was loaded in each lane. KO is represented with Tg Prnp^{-/-} background mice, and is a negative control. All abbreviations for the transgenic lines can be found in the previous figure legend. Membranes were probed with mouse monoclonal Ab 5C6. (B) From Bartz Lab. 500μg brain was loaded in each lane. Each lane is representative brain homogenate of hamster, mink, ferret, squirrel monkey or cow.
**Figure 2.6 Prion protein sequence alignment of various mammals and shuffle PrP #68 in order to assess 5C6 epitope.** Numbers at top indicate mouse number amino acid sequence. The black bar at the bottom indicates secondary structure, $\beta_1$, $\alpha_1$ and partial $\beta_2$ sequentially. Light blue shows one epitope of 5C6 at residue 132 in MoPrP, a polymorphism for ovine PrP. The unique amino acid valine from sheep (VRQ) is labeled circled in red. The other epitope of 5C6 at residue 158 in MoPrP is indicated by royal blue. The unique amino acid, serine, from squirrel monkey PrP, is circled in red.
2) **Monoclonal Antibody 9E9**

We also used 10% brain homogenates from Tg mice expressing various PrP\(^C\) sequences to assess the epitope of the mAb 9E9. Compared with 6H4 in Figure 2.4, the mAb 9E9 reacted with 10% brain homogenates from Tg mice expressing deer PrP, elk PrP, bovine PrP, sheep PrP, and has weak binding to equine PrP. MAb 9E9 did not react with either mouse PrP, human PrP or Tg (M CerB+C) (Figure 2.7). When we aligned the PrP\(^C\) sequences from various species, we found two possible epitope regions for mAb 9E9. One is located before the C2 cleavage site, and the other one is between the C1 and C2 cleavage sites. In order to assess the epitope of mAb 9E9, we removed Asn-linked glycans by treating brain homogenates with \(N\)-glycosidase F (PNGase F). PNGase F is an amidase that cleaves the innermost GlcNAc from asparagine residues on N-linked glycoproteins. If the epitope of 9E9 is located before the C2 cleavage site, we expected to detect the full length of PrP with 9E9 after PNGaseF treatment. If the epitope of 9E9 is located between the C1 and C2 cleavage sites, we expected to detect the full length and C2 fragment of PrP with 9E9 after PNGaseF treatment. To detect the PrP fragment after treatment with PNGase F, we used mAb 6H4 as a control. MAb 6H4’s epitope lies between amino acid residues 144 and 152. We expected it would recognize full-length PrP, 21 kDa C2 fragment, and 17kDa C1 fragment after treatment (Figure 2.8A). In the western blot analysis, mAb 9E9 recognized full length PrP and the 21 kDa C2 fragment, but not the 17 kDa C1 fragment (Figure 2.8B). According to the western blot analysis results, we confirmed 9E9 has no reactivity with mouse PrP and we
narrowed down the epitope of mAb 9E9 to the C1 and C2 endoproteolytic cleavage sites from amino acid 89 to 109 in mouse numbering shown in the box (Figure 2.9). Compared to the amino acids sequence of various species, mAb 9E9 recognizes CerPrP sequence between C1 and C2 cleavage site but fails to recognize MoPrP due to the insertion of a glycine at positions 93 of MoPrP (Figure 2.9). 9E9 has weak binding reactivity with horse and sheep PrP compared to 6H4. Both horse and sheep PrP have a unique amino acid serine, at position 94 as compared to the other mammals which react with 9E9. This might influence the reactivity of 9E9 with horse and sheep PrP.
Figure 2.7 Western blot analysis of assessing 9E9 epitope via brain homogenate of Tg mice. 40 µg transgenic mice brain homogenate was loaded in each lane. KO is represented with Tg Prnp<sup>-/-</sup> background mice, and is a negative control. Tg(MoPrP)4112 shows no reactivity with 9E9, nor does the chimeric Tg(MCerB+C) or the human lines, Tg(HuPrP-M129) and Tg(HuPrP-V129). Tg(DeerPrP), Tg(ElkPrP) and Tg(BoPrP) show reactivity with 9E9. Tg(OvPrP-ARQ), Tg(OvPrP-VRQ) and Tg(EqPrP) show reduced 9E9 binding.
Figure 2.8 Western blot analysis of effect of PrP processing on mAb reactivity. Samples were either untreated (-) or treated (+) with PNGase F or proteinase K as indicated. FVB is a wild type mouse with endogenous PrP. Infected FVB is wild type mouse adapted RML. Tg(ElkPrP) is transgenic line 5037, expressing elk PrP in knockout background mouse. Infected Tg(ElkPrP) was Tg(ElkPrP) mouse adapted CWD isolate 092. PNG: PNGase F. PK: proteinase K. F: Full length of PrP; C2: 21kDa C2 fragment; C1: 17kDa C1 fragment. The blot was probed with (A) anti-PrP mAb 6H4 (B) anti-PrP mAb 9E9. The secondary antibody is anti-mouse in 1:5000 dilution. (performed by Eri Saijo)
Figure 2.9 Prion protein sequence alignment of various mammals and shuffle PrP #68 in order to assess 9E9 epitope. Numbers at top indicate mouse number amino acids sequence. The proteolytic cleavage sites are indicated with arrows. C1 cleavage site is between 110/111. C2 cleavage site is between 88/89. According to the western blot analysis of treatment with PNGase F, we narrowed down the epitope of mAb 9E9 as located between C1 and C2 cleavage sites. Glycine at position 93 in mouse and human PrP blocks the reactivity with 9E9.
3) Monoclonal Antibody 7H11

To detect the reactivity of mAb7H11, we used the same 10% brain homogenates from Tg mice expressing various species PrP\(^C\). MAb 7H11 reacted with mouse PrP, deer PrP, elk PrP, sheep PrP, equine PrP, human PrP and Tg (McerB+C), but did not react with bovine PrP (Figure 2.10). The binding capacity of mAb 7H11 with Tg(Hu-PrP) on western blot seems weaker than other species. When comparing the bovine PrP sequence with other mammals PrP sequences, bovine PrP has a unique amino acid, glutamic acid, at residue 197 in bovine PrP numbering. Because this glutamic acid is located between two N-linked glycans, we think these two N-linked glycans might have a hindrance effect on the binding ability of 7H11. In order to detect the effect between the reactivity of mAb 7H11 and N-linked glycans, un-infected and infected Tg mouse brain homogenates were subjected to PNGase F or proteinase K treatment. For infected brain homogenates treated with PK, we expected to see three bands; Di-glycosylated form, mono-glycosylated form and un-glycosylated form PrP. Comparing the lane loaded with infected brain homogenate treated with PK from Figures 2.11 A and B, we can observe that mAb 6H4 recognized three forms of PrP but mAb 7H11 failed to recognize the di-glycosylated form of PrP from infected brain homogenates after PK treatment. This result indicates that the reactivity of mAb 7H11 is blocked by the presence of N-linked oligosaccharides. The weak reactivity between mAb 7H11 and Tg(HuPrP) brain homogenate, is likely due to the presence of histidine in the human residue at the site homologous to residue 166 in bovine. In the other species, as well as our shuffled immuogen, this residue is tyrosine. Also, the amino acid of interest, glutamic acid, is located
thirty amino acids downstream of the sequence in the disulfide bond region. We think the disulfide bond might be involved in the binding reactivity of mAb 7H11.

To assess the effect of the disulfide bond, two sets of brain homogenate samples were prepared. One set of samples was mixed with loading buffer containing reducing agent (2-Mercaptoethanol) the other set without. Surprisingly, mAb 7H11 had a stronger reaction with the samples lacking the reducing agent (Figure 2.12). Based on these two observations, I hypothesize the epitope of mAb 7H11 is conformational and is sterically hindered by N-linked oligosaccharides. To elucidate my hypothesis, I prepared four samples treated with reducing agent and/or PNGase F. The presence of reducing agent did have an effect on mAb 7H11 binding. Samples that were not treated with reducing agent had a stronger reactivity with mAb 7H11 (Figure 2.13 A and B). Removing the N-linked oligosaccharides via PNGase F (Fig2.13 C and D) had no effect on 7H11 binding capacity.
Figure 2.10 Western blot analysis of assessing 7H11 epitope via brain homogenate of Tg mice. Each lane was loaded with 50µg transgenic mice brain homogenate. KO is represented with Tg Prnp<sup>-/-</sup> background mice, and is a negative control. 7H11 reacted with mouse PrP, deer PrP, elk PrP, sheep PrP, equine PrP, and Tg (McerB+C), weakly with human PrP and did not react with bovine PrP. This blot is probed with hybridoma cell supernatant 7H11. The secondary antibody is anti-mouse in 1:5000 dilution.
**Figure 2.11 Effect of PrP processing on mAb 7H11 reactivity.** Samples were either untreated (-) or treated (+) with PNGase F or proteinase K as indicated. FVB is a wild type mouse with endogenous PrP. Infected FVB is wild type mouse adopted RML. Tg(ElkPrP) is transgenic line 5037, expressing elk PrP in knockout background mouse. Infected Tg(ElkPrP) was Tg(ElkPrP) mouse adapted CWD isolate 092. PNG: PNGase F. PK: proteinase K. Di-: di-glycosylated form PrP; Mono-: mono-glycosylated form PrP; Un-: un-glycosylated form PrP. The blot was probed with (A) anti-PrP mAb 6H4 (B) anti-PrP mAb 7H11. The secondary antibody is anti-mouse in 1:5000 dilution. (performed by Eri Saijo)
Figure 2.12 Effect of reducing agent on 7H11 binding.

In order to see the effect of reducing agent on 7H11 binding, samples were treated with or without beta-Mercaptoethanol. (A) Samples were not treated with beta-Mercaptoethanol and (B) Samples were treated with beta-Mercaptoethanol. KO refers to mice in which the prnp coding sequence is disrupted and express no PrP\(^{\text{C}}\), and is a negative control. All other transgenic lines are as previously described. 7H11 binding was stronger under non-reducing conditions. 10ul from 10% brain homogenate
Figure 2.13 Effect of PrP processing and reducing agent on mAb 7H11 reactivity. In order to see the effect of reducing agent and glycosylation site, samples were treated with reducing agent beta-Mercaptoethanol or PNGase F. KO refers to mice in which the prnp coding sequence is disrupted and express no PrP\textsuperscript{C}, and is a negative control. FVB is indicated wild type mouse brain homogenate. Tg(DeerPrP) is transgenic line 1536, expressing deer Prion in mouse. Tg(ElkPrP) is transgenic line 5037, expressing elk PrP\texttextsuperscript{C} in knockout background mouse. Tg(BoPrP) is transgenic line 3705, expressing bovine PrP\texttextsuperscript{C} in knockout background mouse. Tg(HuPrP-M129) is transgenic line 6816, expressing human PrPC with amino acid methionine in 129 in knockout background mouse. Tg(HuPrP-V129) is transgenic line 7826, expressing human PrP\texttextsuperscript{C} with amino acid valine in 129 in knockout background mouse. S.Hamster is syrian hamster brain homogenate, 10\mu l from 10% brain homogenate (A) No treatment (B) samples were treated with beta-Mercaptoethanol (C) samples were treated with PNGase F only. (D) Samples were treated with beta-Mercaptoethanol and PNGase F. These blots were probed with 7H11.
Figure 2.14 Prion protein sequence alignment of various mammals and shuffle PrP #68 in order to assess 7H11 epitope. Numbers at top indicate mouse number amino acids sequence. The glycosylation sites are designated by green hexagons, and highlighted in green boxes. The black bars at the bottom indicate secondary structure, partial α-helix 1, β-sheet 2, α-helix 2 and partial α-helix 3 sequentially. Disulfide bridge which linked α-helix 2 and α-helix 3 is indicated in red lines. According to the western blot analysis, mAb 7H11 failed to recognize bovine PrP (cow) which has a unique amino acid is located between two N-linked glycosylation sites. Amino acids labeled with red circle indicate the potential epitope of 7H11.
4) Monoclonal Antibody 9H9

In western blots analysis, the mAb 9H9 reacted with 10% Tg mouse brain homogenates expressing mouse PrP and Tg (McerB+C), but did not react with cervid PrP, bovine PrP, ovine PrP, equine PrP and human PrP (Fig 2.15A). MAb 9H9 also recognized PrP in Syrian hamster brain homogenate, but did not react with mink PrP, ferret PrP, squirrel monkey PrP and cow PrP (Figure 2.15B, performed by Dr. Jason Bartz lab). Based on the western blot analysis of Tg mouse homogenates and brain homogenates of other mammals, mAb 9H9 is specific to mouse and Syrian hamster PrP. Comparing the sequences of various mammals and shuffled PrP #68, we conclude that the epitope of mAb 9H9 is at the C-terminus of mouse PrP (Fig 2.16).

Table 2 shows the summary of our in-house monoclonal antibodies reactivity with various species.
Figure 2.15 Western blot analysis of assessing 9H9 epitope via brain homogenate of (A) Tg mice and (B) mammals. (A) 50 ug transgenic mice brain homogenate was loaded in each lane. This blot is probed with 9H9. The secondary antibody is anti-mouse in 1:5000 dilution. Reactivity is seen with mouse and chimeric PrP only. (B) This blot was done by Dr. Jason Bartz Lab. 500ug brain was loaded in each lane. Each lane is represented hamster, mink, ferret, squirrel monkey and cow sequentially. Only hamster shows reactivity.
### Figure 2.16 Prion protein sequence alignment of various mammals and shuffled PrP #68 in order to assess 9H9 epitope.

Numbers at top indicate mouse sequence number. According to western blot analysis, mAb 9H9 only has reactivity with mouse and Syrian hamster. Blue box is showing the unique sequence in mouse and hamster is same as our immunogen, shuffled PrP #68.

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<th>Sequence</th>
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<tr>
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226-230 in mouse sequence
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<td>Squirrel Monkey</td>
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</table>

"-" indicates no reactivity. "+" indicates positive reactivity. ND indicates not determined.
Section 2: Epitope tagging

As described in the introduction, pathogenesis in prion diseases is mediated by abnormal folding of prion protein from alpha-helix rich form PrP\textsuperscript{C} to beta-sheet rich form PrP\textsuperscript{Sc}. Unfortunately, the mechanism of conversion from PrP\textsuperscript{C} to PrP\textsuperscript{Sc} is still unclear. Since we have generated these novel monoclonal antibodies, we can utilize them, in conjunction with epitope tagging, to track PrP\textsuperscript{C} trafficking which could elucidate the conversion process. We also can confirm these monoclonal antibodies epitopes by insertion or elimination of the epitopes predicted in section 1. All mutated PrPs were created via mutagenesis and transfected into rabbit kidney epithelial cells (RK13 cell) which do not express endogenous PrP\textsuperscript{C}. Table 3 shows a summary of the constructs we made derived from elk, mouse, and bovine PrP and their reactivity to our in-house monoclonal antibodies.
Table 3. Summary of constructs derived from elk, mouse, and bovine PrP and their reactivity to various monoclonal antibodies.

<table>
<thead>
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<td></td>
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<td>Δ93G</td>
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<td>N/A</td>
<td>Q185E</td>
</tr>
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</tbody>
</table>

Table 3. The top row indicates the PrP species from which the constructs are derived. The second row indicates positive (+) or non (-) reactivity of the constructs to the mouse monoclonal antibodies indicated on the left (5C6, 9E9, 7H11, 9H9). ‘Wild type’ indicates the original PrP of the species in the corresponding column. ‘N/A’ indicates data not available. Substitution mutant nomenclature is as follows: ‘A133V’ indicates that Alanine in 133rd position in elk PrP is substituted with Valine. Addition of a residue is described as follows: ‘93G’ indicates that a Glycine was inserted after the 93rd position in elk PrP. Deletion of a residue is described as follows: ‘Δ93G’ indicates that a Glycine was deleted from the 93rd position of mouse PrP.
A. The substitution of residue 132 in mouse PrP affects the susceptibility of RML infection in cell culture model

Mutating the proposed amino acids of the 5C6 epitope is one way to confirm that these amino acids are required for 5C6 recognition. As described in result section 1, the epitope of mAb 5C6 is located at amino acids 132 alanine and 158 asparagine (in mouse numbering) (Figure 2.6). In order to differentiate various PrPs we eliminated the 5C6 epitope by mutating the alanine to valine at residue 132, or asparagine to serine at residue 158 in mouse PrP. These constructs are referred to as MoPrP-A132V and MoPrP-N158S. The corresponding amino acids were mutated in elk PrP to eliminate the 5C6 epitope: alanine to valine at residue 133 or asparagine to serine at residue 162. These constructs are referred to as ElkPrP-A133V and ElkPrP-N162S. To generate stable cell lines expressing PrPs of interest, RK13 cells were transfected with wild type mouse PrP, MoPrP-A132V, MoPrP-N158S, wild type elk PrP, elkPrP-A133V or elkPrP-N162S cloned into a pIRES-puro3 vector. All cell lines expressing a PrP\(^\text{C}\) with a mutated 5C6 epitope could be detected by mab 6H4 (Figure 3.1A). However, only wild type PrP, but not mutated PrPs could be detected by mAb 5C6 (Figure 3.1B). These results demonstrate that the mutated constructs were successfully expressed in the RK13 cells and that mutation of either alanine or asparagine is sufficient to abolish 5C6 recognition. It also confirmed the epitope of mAb 5C6 we described in section 1.

In order to determine susceptibility of the mutated mouse PrP or elk PrP in RK 13 cells to prions, we infected these cells with appropriate strains: mouse-adapted
RML scrapie prions in the case of cells expressing MoPrP variants and CWD isolate bala05 in the case of cells expressing elkPrP variants. The infected cultures were passaged three times in order to reduce detectable PrP\textsuperscript{Sc} from infection. Previous experiment showed infection of cells with RML or CWD resulted in detectable PrP\textsuperscript{Sc}, however, there is a progressive reduction of PrP\textsuperscript{Sc} after repeated passage (Bian, Napier et al. 2010). To analyze mouse PrP\textsuperscript{Sc} and elk PrP\textsuperscript{Sc} by western blotting, detergent extracts containing equal amounts of protein were treated with PK and centrifuged for 1 hour at 100,000 x g. This high speed centrifugation pelleted insoluble PrP\textsuperscript{Sc} molecules. We used proteinase K to detect the PrP\textsuperscript{Sc} formation in culture. In mouse cell lines, we detected PrP\textsuperscript{Sc} molecules in wild type mouse PrP after treating cultures with mouse-adapted RML scrapie prions but not in the RK13 cells expressing empty pIRES vector. Mouse PrP\textsuperscript{C} with an amino acid switch from asparagine to serine does not affect the conversion ability from mouse PrP\textsuperscript{C} to mouse PrP\textsuperscript{Sc}. However substituting alanine to valine at residue 132 in mouse PrP affects the susceptibility to RML (Figure 3.2). In elk cell lines, no PrP\textsuperscript{Sc} was detected in mutated elk PrP constructs but wild type elk PrP (Figure 3.3) did show PrP\textsuperscript{Sc} formation. Substituting alanine with valine in mouse PrP at position 132 affects mouse PrP\textsuperscript{Sc} formation and substituting the same position in elk PrP also affects elk PrP\textsuperscript{Sc} formation. The residue 132 in mouse PrP corresponding to residue 136 in sheep PrP is a polymorphism in sheep PrP. A previous report showed that sheep with valine at residue 136 had higher susceptibility to scrapie compared to sheep with alanine at residue 136 (Acin, Martin-Burriel et al. 2004). This indicated that the alanine at residue 132 in mouse PrP might be involved in the conversion of PrP\textsuperscript{C}.
to PrP\textsuperscript{Sc} and also implicated it as a residue responsible for the species barrier between mouse and sheep. Substituting asparagine with serine in mouse PrP at position 158 does not affect mouse PrP\textsuperscript{Sc} formation but substituting the same position in elk PrP affects elk PrP\textsuperscript{Sc} formation. This indicated these residues are species specific.
Figure 3.1 Western blot analysis of RK13 cells expressing mutated elk or mouse PrP to assess 5C6 reactivity. To assess the 5C6 epitope, the potential epitope was introduced or eliminated from elk or mouse constructs. The constructs were cloned into pIRES-Puro3 and transfected into rabbit kidney cells (RK13) that do not express endogenous PrP. RK-Vector is negative control expressing pIRES vector only. RK-MoPrP denotes RK13 cells expressing full length of mouse PrP. RK-ElkPrP denotes RK13 cells expressing full length elk PrP. RK-ElkPrP-A133V denotes RK13 cells expressing elk PrP with substitution alanine to valine at residue 133. RK-ElkPrP-N162S denotes RK13 cells expressing elk PrP with substitution arginine to cystine at residue 162. RK-MoPrP-A132V denotes RK13 cells expressing mouse PrP with substitution alanine to valine at residue 132. RK-MoPrP-N158S denotes RK13 cells expressing mouse PrP with substitution arginine to cystine at residue 158. Membranes were probed with (A) mouse monoclonal Ab 6H4 at 1:10,000 dilution and (B) mouse monoclonal Ab 5C6 at 1:20 dilution. Secondary antibody: HRP-conjugated anti-mouse (GE) at 1:5,000 dilution. Blots were developed with chemiluminescence (GE) and visualized via Fuji scanner.
Figure 3.2 Western blot analysis of conversion ability of mutated mouse PrP expressed in RK13 cells. To detect the ability of PrP<sup>C</sup> to PrP<sup>Sc</sup> conversion in mutated mouse PrPs, the cells were infected with RML brain homogenate. RKV is negative control expressing pIRES vector in RK13 cells. RKM denotes RK13 cells expressing full length of mouse PrP. RKM-A132V denotes RK13 cells expressing mouse PrP with substitution alanine to valine at residue 132. RKM-N158S denotes RK13 cells expressing mouse PrP with substitution arginine to cystine at residue 158. The first six lanes are the cell lines without infection. All lines were challenged with 0.1% RML brain homogenate. Cell lysates were collected with cold lysis buffer from 10 cm culture plate at passage 3. Total protein content was measured by bicinchoninic acid assay (BCA). Cell lysates detergent extracts containing equal amounts protein were treated with 30 mg/ml PK for 1 hr at 37°C and centrifuge for 1 h at 100,000 x g. Membranes were probed with mouse monoclonal Ab 6H4 at 1:10,000 dilution. Secondary antibody: HRP-conjugated anti-mouse (GE) at 1:5,000 dilution. Blots were developed with chemiluminescence (GE) and visualized via Fuji scanner.
Figure 3.3 Western blot analysis of conversion ability of mutated elk PrP in RK13 cells. To detect the ability of PrP\textsuperscript{c} to PrP\textsuperscript{Sc} conversion in new construct, the cells were infected with CWD brain homogenate isolate Bala 05. RKV is negative control expressing pIRES vector in RK13 cells. RKE denotes RK13 cells expressing full length elk PrP. RKE-A133V denotes RK13 cells expressing elk PrP with substitution alanine to valine at residue 133. RKE-N162S denotes RK13 cells expressing elk PrP with substitution arginine to cystine at residue 162. The first six lanes show the cell lines without infection. Those cell lines indicated were infected with 1/\% CWD brain homogenate isolate Bala 05. Cell lysates were collected with cold lysis buffer from 10 cm culture plate at passage 3. Cell lysates were collected with cold lysis buffer from 10 cm culture plate at passage 3. Total protein content was measured by bicinchoninic acid assay (BCA). Cell lysates detergent extracts containing equal amounts protein were treated with 30 mg/ml PK for 1 hr at 37\(^\circ\)C and centrifuge for 1 h at 100,000 \(\times\) g. Membranes were probed with mouse monoclonal Ab 6H4 at 1:10,000 dilution. Secondary antibody: HRP-conjugated anti-mouse (GE) at 1:5,000 dilution. Blots were developed with chemiluminescence (GE) and visualized via Fuji scanner.
B. The deletion of residue 93 in mouse PrP affects the susceptibility of RML infection in cell culture model

Based on the previous western blot analysis, mAb 9E9 is specific to the C2 fragment and has narrow specificity for cervid PrP (Figure 2.9). The epitope for 9E9 appears to be comprised of GQGGTH in elk and deer PrP. Mouse PrP at the same location has the sequence of GQGGGTH. There is one extra glycine in mouse PrP. To confirm that this epitope constituted the 9E9 recognition sequence we introduced an extra G in elk PrP and eliminated the corresponding G in MoPrP via mutagenesis. We supposed that glycine at residue 93 in mouse PrP interrupts 9E9's binding with mouse PrP. We successfully transfected and expressed all wild type PrPs and variants in RK13 cells as seen via blotting with mAb 6H4 (Figure 3.4A). All PrP\textsuperscript{C} can be detected by mAb 6H4 indicating all successfully. Mouse PrP with one glycine deletion at position 93 was recognized by mAb 9E9, and elk PrP with one glycine insertion at same position did not react with mAb 9E9 (Figure 3.4B). This result showed that this extra glycine in mouse PrP inhibits 9E9 recognition.

We also would like to determine the susceptibility of the established cell lines to prions. The cell lines expressing elk and mouse PrP\textsuperscript{C} and their mutated counterparts were also infected with appropriate strains in order to assess the ability of mutated PrP\textsuperscript{C} to be converted to PrP\textsuperscript{Sc}. RML inoculums were used to infect cells expressing mouse PrP and its mutant, and CWD inoculums were used to infect cells expressing cervid PrP and its mutant. All the samples were prepared as described in previous studies. Consistent with the 5C6 result in
mutated elk cell lines, we did not observe PrP\textsuperscript{Sc} from cells expressing elk PrP with the insertion of glycine but did see conversion in wild type elk PrP (Figure 3.5). However, we did detect the PrP\textsuperscript{Sc} from cells expressing mutated mouse PrP containing 9E9 epitope and wild type mouse PrP (Figure 3.6). Interestingly, we could not detect the diglycosylated form of PrP\textsuperscript{Sc} in the mutated mouse PrP which contained the 9E9 epitope. These results demonstrated that an extra glycine in mouse PrP sequence is sufficient to interrupt 9E9 recognition. Producing 9E9 epitope by deleting this one extra glycine in mouse PrP does not prevent formation of mouse PrP\textsuperscript{Sc} but decreases formation of di-glycosylated PrP\textsuperscript{Sc}. Inserting one extra glycine at position 93 in elk PrP both interferes with 9E9 recognition and affects elk PrP\textsuperscript{Sc} formation.
Figure 3.4 Western blot analysis of RK13 cells expressing mutated elk or mouse PrP to assess 9E9 reactivity To assess the 9E9 epitope, the potential epitope was introduced or eliminated from elk or mouse constructs. The constructs were cloned into pIRES-Puro3 and transfected into rabbit kidney cells that do not express endogenous PrP. RK-Vector is negative control expressing pIRES vector only. RK-MoPrP denotes RK13 cells expressing full length of mouse PrP. RK-ElkPrP denotes RK13 cells expressing full length elk PrP. RK-ElkPrP-93G denotes RK13 cells expressing elk PrP with a glycine after residue 93. RK-MoPrP-Δ93G denotes RK13 cells expressing mouse PrP with glycine deletion at residue 93. Membranes were probed with (A) mouse monoclonal Ab 6H4 at 1:10,000 dilution and (B) mouse monoclonal Ab 9E9 at 1:5 dilution. Secondary antibody: HRP-conjugated anti-mouse (GE) at 1:5,000 dilution. Blots were developed with chemiluminescence (GE) and visualized via Fuji scanner.
**Figure 3.5 Western blot analysis of conversion ability of mutated elk PrP in RK13 cells.** To assess the ability of PrP\textsuperscript{C} to convert to PrP\textsuperscript{Sc}, the transfected cells were infected with CWD brain homogenate isolate Bala 05. RK-V is negative control expressing pIRES vector in RK13 cells. RKE denotes RK13 cells expressing full length elk PrP. RKE-93G denotes RK13 cells expressing elk PrP with a glycine after residue 93. The first four lanes indicate cell lines without CWD infection. Those cell lines indicated were infected with 1/\% CWD brain homogenate isolate Bala 05. Cell lysates were collected with cold lysis buffer from 10 cm culture plate at passage 3. Total protein content was measured by bicinchoninic acid assay (BCA). Cell lysates detergent extracts containing equal amounts protein were treated with 30 mg/ml PK for 1 hr at 37\textdegree{}C and centrifuge for 1 h at 100,000 x g. Membranes were probed with mouse monoclonal Ab 6H4 at 1:10,000 dilution. Secondary antibody: HRP-conjugated anti-mouse (GE) at 1:5,000 dilution. Blots were developed with chemiluminescence (GE) and visualized via Fuji scanner.
Figure 3.6 Western blot analysis of conversion ability of mutated mouse PrP in RK13 cells. To detect PrP \textsuperscript{C} to PrP \textsuperscript{Sc} conversion in new constructs, the cells were infected with transgenic mice adapted RML brain homogenate. The constructs were cloned into pIRES-Puro3 and transfected into rabbit kidney cells that do not express endogenous PrP. RKV is negative control expressing pIRES vector in RK13 cells. RKM denotes RK13 cells expressing full length of mouse PrP. RKM-Δ93G denotes RK13 cells expressing mouse PrP with glycine deletion at residue 93. The first four lanes indicate cell lines without RML infection. Those cell lines indicated were infected with 0.1\% RML brain homogenate. Red square indicates the location of diglycosylated PrP. Cell lysates were collected with cold lysis buffer from 10 cm culture plate at passage 3. Total protein content was measured by bicinchoninic acid assay (BCA). Cell lysates detergent extracts containing equal amounts protein were treated with 30 mg/ml PK for 1 hr at 37\textdegree C and centrifuge for 1 h at 100,000 \times g. Membranes were probed with mouse monoclonal Ab 6H4 at 1:10,000 dilution Secondary antibody: HRP-conjugated anti-mouse (GE) at 1:5,000 dilution. Blots were developed with chemiluminescence (GE) and visualized via Fuji scanner.
C. Confirmation of potential epitope of mAb 7H11 and mAb 9H9

In section 1, we found 7H11 could not recognize bovine PrP and has weaker reactivity with human PrP. We also found the binding ability of 7H11 was affected by N-linked oligosaccharides and disulfide bridge. Based on these results, we hypothesized the epitope of mAb 7H11 is located at residue 166 and 197 of BoPrP. To further ascertain the recognition site of mAb 7H11, we mutated BoPrP at residue 197 from glutamic acid to glutamine. This construct was referred to BoPrP-E197Q. This residue is unique in bovine PrP sequence compare to other mammals PrP sequence and our immunogen. The corresponding amino acids were also mutated from glutamine to glutamic acid at residue 185 in mouse PrP to eliminate the 7H11 epitope. This construct was referred to MoPrP-Q185E. These two mutated constructs were cloned into pIRES-Puro3 vector and used to generat cell lines in RK13 cells. Bovine PrP-E197Q and mouse PrP-Q185E were examined via western blot with mAb 7H11. As expected, mAb 7H11 lost its affinity for mouse PrP when the substitution at 185 was made. Mutating Bovine PrP to the mouse sequence at residue 197 (bovine numbering) did not confer 7H11 binding. Ongoing studies are investigating the importance of amino acid 166 (bovine numbering) in the ability of mAb 7H11 to recognize Bovine PrP.

In the previous mapping results, the epitope of mAb 9H9 is located at the C-terminus of mouse PrP (Figure 2.16). The unique amino acid sequence of mouse and Syrian hamster PrP in the C-terminus is DGRRS which coincides with the immunogen used to generate our antibodies, shuffled PrP#68. In the same
region of other mammals’ PrPs, the amino sequence is QRG. To clarify the binding site of 9H9 and generate a cervid cell line that could be recognized by mAb 9H9, three mutant cervid RK13 cell lines were generated by mutating QR to DGRR, RR or DGQR starting from residue 230 based on the alignment of mammalian PrP. These constructs are referred to as ElkPrP-QR230DGRR, ElkPrP-QR230RR, and ElkPrP-QR230DGQR. We substituted residue 230Q and 231R in elk PrP sequence to DGRR to make the sequence the same as mouse PrP and our immunogen. To define the 9H9 recognition site more precisely, two other constructs were generated, ElkPrP-QR230RR, and ElkPrP-QR230DGQR. All mutated constructs and wild type were expressed successfully in RK13 cells as seen via western blotting with mAb 6H4 (Figure 3.7A). Only one mutant, QR230DGRR, showed reactivity with mAb 9H9, but it was extremely weak (Figure 3.7B). In figure 3.7A, mutated elk PrPs have lower expression levels as compared to wild type elk PrP. In our previous experience, mAb 9H9 has low affinity with cell culture samples. The low expression level of the mutated elk PrPs in RK13 cells coupled with 9H9's low affinity with cell culture samples could explain the weak reactivity seen in figure 3.7B. However, it is also possible that there are other amino acids which might be involved in the reactivity of 9H9. The serine in the extreme C-terminus might be necessary for 9H9 binding.
Figure 3.7 Western blot analysis of RK13 cells expressing mutated elk or mouse PrP to assess 9H9 reactivity. Monoclonal antibody 9H9 has a specific reactivity with mouse and Syrian hamster. To assess the 9H9 epitope, the proposed epitope was introduced into elk constructs. The constructs were cloned into pIRES-Puro3 and transfected into RK13 rabbit kidney cells that do not express endogenous PrP. RK-Vector indicates negative control expressing pIRES vector in the RK13. RK-ElkPrP denotes RK13 cells expressing full length of elk PrP. RK-ElkPrP-QR230DGRR denotes RK13 cells expressing elk PrP with amino acids substitution QR with DGRR from residue 230. RK-ElkPrP-QR230RR is RK13 cell expressing elk PrP with amino acids substitution with QR with RR from residue 230. RK-ElkPrP-QR230DGQR denotes RK13 cell expressing elk PrP with amino acids substitution QR with DGQR from residue 230. Membranes were probed with (A) mouse monoclonal Ab 6H4 at 1:10,000 dilution and (B) mouse monoclonal Ab 9H9. Secondary antibody: HRP-conjugated anti-mouse (GE) at 1:5,000 dilution. Blots were developed with chemiluminescence (GE) and visualized via Fuji scanner.
Section 3: Structure analysis via deletion

Despite having the same sequence, PrP<sup>C</sup> and PrP<sup>Sc</sup> are quite different both structurally and biochemically. To elucidate the mechanism of conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup> has been a long-standing goal in prion disease research. Conformational change from PrP<sup>C</sup> to PrP<sup>Sc</sup> results in a higher percentage of beta sheet structure and increased protease resistance. In an attempt to acquire information from PrP’s primary structure regarding conformational changes during conversion, we generated a series of ten contiguous amino acids deletion constructs spanning amino acids 107 to 230 to find out which region is involved in the conformational change (Figure 4.1). Amino acids 107 to 230 in mouse PrP is a region containing secondary structure and post-modifications. I used these previously generated deletion constructs for mapping our novel monoclonal antibodies. Figure 4.1 shows the scheme of the serial deletion constructs we made and the location of glycosylation sites and cysteines involved in disulfide bonding. Also it indicates what secondary structure is lacking in each deletion construct. All the deletion constructs were made via mutagenesis and cloned into pIRES-puro3 vector (Invitrogen). These constructs were transfected into RK13 cells and expression was checked via immunoblotting with monoclonal antibody R1 (Figure 4.2). R1 was generated by immunization with PrP liposomes and recognizes residues 225 to 230 in the C-terminus of PrP (Williamson, Peretz et al. 1998). In other words, R1 recognized all the deletion constructs except MoPrPΔ222-230 which includes recognition site of R1. One of the N-linked oligosaccharide sites is in the deletion mutant 192-201 and has been
removed; therefore this mutant has lower molecular weight than other deletion mutants. When we analyzed these cell lines with mAb 6H4, not only the deletion which encompasses its epitope site, but also other deletions in the central region of construct lacked reactivity (Figure 4.3). Most of the deletions from 141 to 221 have no reactivity with mAb 6H4, Only MoPrP\(\Delta\)182-191 and MoPrP\(\Delta\)192-201 have very weak reactivity. Based on sections 1 and 2, we know the epitope of mAb 5C6 is at residue 132 and 158 of MoPrP. Therefore, we expected there to be no reactivity with MoPrP\(\Delta\)132-141 or MoPrP\(\Delta\)152-161. Surprisingly, constructs with deletions ranging from 121 to 221 showed no recognition with mAb 5C6 (Figure 4.4). We continued to analyze our in-house monoclonal antibodies with these constructs expressing in RK 13 cells. MAb 9E9 is does not detect mouse PrP, so we did not use for analysis. The 7H11 epitope is sterically hindered by N-linked oligosaccharides and 7H11 binding is increased in the presence of the disulfide bond. 7H11 has two epitopes, residue 154 and 185 (in mouse numbering) (Figure 2.14). We observed hindrance effects in MoPrP\(\Delta\)141-151 to MoPrP\(\Delta\)211-221 with 7H11 (Figure 4.5). As we expected, MAb 9H9 recognized all the deletion constructs excepting MoPrP\(\Delta\)222-230 which contains the 9H9 epitope. Only antibodies with epitopes in the C-terminus of PrP meet our expectation that the antibody will recognize all deletions except for that which encompasses its epitope.

We wondered if post-translational modifications in mammalian cells could influence the antibody binding capacity in our deletion constructs. To address this question, we transferred all deletion constructs into pET-100 vector and
expressed them in a bacterial system. In the bacterial system, there is no post-translational modification, but proteins do form secondary structure and disulfide bonds. Since the 6H4 epitope lies somewhere between amino acids 144 and 152 we hypothesized that the deletion mutants 142-151 and 152-161 would not be recognized by 6H4. As we expected, mAb 6H4 recognized all the constructs excepted for deletions including the binding region in the bacterial system (Figure 4.7). However, when we detected these constructs with other antibodies, we observed an epitope shielding phenomenon, as in the mammalian system. In the western blot, most of the deletion constructs with deletions from the central region of 132 to 221 had no reactivity with mAb 5C6 (Figure 4.8). Only MoPrPΔ182-191 had slight reactivity with 5C6. When analyzing mAb 7H11 with these constructs in bacterial system, 7H11 recognized wild type recombinant mouse PrP and some deletion constructs but did not have reactivity with the constructs containing deletions close to the 7H11 epitope (Figure 4.9). MAb 9H9 recognized wild type PrP and all the deletion constructs except MoPrPΔ222-230, just as in the mammalian cells (Figure 4.10). However, the binding reactivity of 9H9 with recombinant protein is stronger than it is with mammalian cell lysates. Comparing western blots probed with 9H9 or other monoclonal antibodies, we confirmed the binding site of 9H9 is located in the C-terminus of PrP. Since mAb 5C6 recognizes alanine at position 132 and asparagines at position 158 in mouse PrP, it is a conformation dependent monoclonal antibody, as is 7H11. Routinely, before performing gel electrophoresis, all samples are mixed with denaturing reagents like sodium dodecyl sulfate (SDS), an anionic detergent which denatures secondary and non–disulfide–linked tertiary structures, and applies a
negative charge to each protein in proportion to its mass. Traditional wisdom dictates that conformation dependent monoclonal antibodies cannot detect proteins which are denatured. However, in our western blot results, we can detect wild type mouse PrP with our conformation dependent monoclonal antibodies, 5C6 and 7H11. That indicates that the structured region of PrP forms a very stable structure that cannot be denatured by a detergent like SDS. The epitopes of 9H9 or R1 are in the unstructured C-terminus region, which can be recognized.
Figure 4.1 The scheme of the serial deletion constructs
**Figure 4.2 Western blot analysis of serial deletion constructs in mammal cell line with R1.** All deletion constructs were cloned into pIRES-Puro3 and transfected into RK13 cells. Cell lysates were collected with cold lysis buffer from 10 cm culture plate. Total protein content was measured by bicinchoninic acid assay (BCA). Each well was loaded 50µg cell lysate. Membrane was probed with anti-PrP R1 at 1:10,000 dilution which epitope is 225 to 230. Secondary antibody: HRP-conjugated anti-human (pierce) at 1:5,000 dilution. Blots were developed with chemiluminescence (GE) and visualized via Fuji scanner.
Figure 4.3 Western blot analysis of serial deletion constructs in mammal cell line with 6H4. All deletion constructs were cloned into pIRES-Puro3 and transfected into RK13 cells. Each well was loaded 50µg cell lysate. Membrane was probed with anti-PrP 6H4 with 1:10000 dilutions which epitope is from 144 to 152. Secondary antibody: HRP-conjugated anti-mouse (GE) at 1:5,000 dilution. Blots were developed with chemiluminescence (GE) and visualized via Fuji scanner.
Figure 4.4 Western blot analysis of serial deletion constructs in mammal cell line with 5C6. All deletion constructs were cloned into pIRES-Puro3 and transfected into RK13 cells. Each well was loaded 50µg cell lysate. Membrane was probed with mouse monoclonal Ab 5C6 at 1:20 dilution. Secondary antibody: HRP-conjugated anti-mouse (GE) at 1:5,000 dilution. Blots were developed with chemiluminescence (GE) and visualized via Fuji scanner.
Figure 4.5 Western blot analysis of serial deletion constructs in mammal cell line with 7H11. All deletion constructs were cloned into pIRES-Puro3 and transfected into RK13 cells. Each well was loaded 75µg cell lysate. Membrane was probed with mouse monoclonal Ab 7H11 without dilution. Secondary antibody: HRP-conjugated anti-mouse (GE) at 1:5,000 dilution. Blots were developed with chemiluminescence (GE) and visualized via Fuji scanner.
Figure 4.6 Western blot analysis of serial deletion constructs in mammal cell line with 9H9. All deletion constructs were cloned into pIRES-Puro3 and transfected into RK13 cells. Each well was loaded 75µg cell lysates. Membrane was probed with mouse monoclonal Ab 9H9 without dilution. Secondary antibody: HRP-conjugated anti-mouse (GE) at 1:5,000 dilution. Blots were developed with chemiluminescence (GE) and visualized via Fuji scanner.
Figure 4.7 Western blot analysis of serial deletion constructs in bacterial system via 6H4. All deletion constructs were cloned into pET-100 and transformed into BL21. Seed 1:50 dilution overnight bacterial broth and grow until OD 600 reach 0.6. Added 1mM IPTG into broth and grew for 4 hours. Collected 1 ml induced bacteria cell lysate and mixed with 100 µl 1X loading buffer. Each well was loaded 5µl cell lysate. Membrane was probed with anti-PrP 6H4 with 1:10000 dilutions which epitope is from 144 to 152. Secondary antibody: HRP-conjugated anti-mouse (GE) at 1:5,000 dilution. Blots were developed with chemiluminescence (GE) and visualized via Fuji scanner.
Figure 4.8 Western blot analysis of serial deletion constructs in bacterial system via 5C6. All deletion constructs were cloned into pET-100 and transformed into BL21. Each well was loaded 5µl cell lysate. Membrane was probed with mouse monoclonal Ab 5C6 with 1:20 dilution. Secondary antibody: HRP-conjugated anti-mouse (GE) at 1:5,000 dilution. Blots were developed with chemiluminescence (GE) and visualized via Fuji scanner.
Figure 4.9 Western blot analysis of serial deletion constructs in bacterial system via 7H11. All deletion constructs were cloned into pET-100 and transformed into BL21. Each well was loaded 5µl cell lysate. Membrane was probed with mouse monoclonal Ab 7H11 without dilution. Secondary antibody: HRP-conjugated anti-mouse (GE) at 1:5,000 dilution. Blots were developed with chemiluminescence (GE) and visualized via Fuji scanner.
Figure 4.10 Western blot analysis of serial deletion constructs in bacterial system via 9H9. All deletion constructs were cloned into pET-100 and transformed into BL21. Lane 1 is expressing pET-Vector in BL21. Each well was loaded 5 µl cell lysate. Membrane was probed with mouse monoclonal Ab 9H9 without dilution. Secondary antibody: HRP-conjugated anti-mouse (GE) at 1:5,000 dilution. Blots were developed with chemiluminescence (GE) and visualized via Fuji scanner.
Prions are transmitted in an unconventional way via a protein particle without nucleic acids. It is still mysterious and many aspects are as yet unknown. Most anti-PrP antibodies are expensive. Conformational change is a major component in the mechanism of prion replication. The two forms of PrP share the same amino acid sequence, but have drastically different biochemical properties. In an attempt to acquire more information on the conversion process, such as key sites, we created novel sequences containing mouse and elk PrP via shuffling of the two species' PrP DNA sequences. In the hopes that we could generate new and novel prion antibodies to answer some of the mysteries surrounding the conversion process, we used three of our shuffled DNA constructs as immunogen for in-house antibody production. These three constructs were selected for producing immunogen, because their sequences are distributed equally between mouse and elk PrP. Using these new PrP sequences which mixed mouse and elk PrP, we generated a series of monoclonal antibodies with novel epitopes. We collected all the hybridoma cells generated by three shuffled PrP with novel sequence. Each shuffled PrP produced thirty to fifty hybridoma cells. We only screened those hybridoma cells generated by shuffled PrP#68. Four novel mAbs have been characterized and each of them has unusual and useful properties. They are 5C6, 9E9, 7H11 and 9H9.
MAb 5C6 reacts efficiently with PrPs from a broad range of mammalian species including mouse, deer, elk, bovine, equine, human, Syrian hamster, mink, ferret, and sheep with alanine in residue 136. According to the sequence of sheep (VRQ) and squirrel monkey, residues 132 alanine and 158 asparagine are necessary for 5C6 binding. We confirmed this when we substituted alanine with valine at position 132 or asparagine with serine at position 158 in mouse PrP and the same position in elk PrP. There are 26 amino acids between these two binding sites. This indicated that 5C6 is a conformation dependent mAb. These two recognition sites span the base of the loop between β1 and β2 and are close to each other when the protein is folded. To investigate the role of the 5C6 epitope, we expressed these mutated mouse PrP variants or mutated elk PrP variants in the RK13 cells and infected with proper prions. Substituting alanine with valine in mouse PrP at position 132 affects mouse PrP<sup>Sc</sup> formation and substituting the same position in elk PrP also affects elk PrP<sup>Sc</sup> formation. Residue 132 in mouse PrP corresponds to residue 136 in sheep PrP. This residue is also one polymorphism of sheep PrP. A previous report showed that sheep PrP with valine at residue 136 has higher susceptibility to scrapie compared to sheep PrP with alanine at residue 136 (Acin, Martin-Burriel et al. 2004). That indicated the alanine at residue 132 in mouse PrP might be involved in the conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup> and might be the amino acid that results in the species barrier between mouse and sheep. To prove this assumption, we can infect the cell line, RK-MoPrPA132, with sheep scrapie. If this cell line can be infected by sheep scrapie, then it indicates residue 132 in mouse PrP is the key site for species barrier between sheep and mouse or other mammals. It is possible because
most other mammals PrP sequence have alanine at that position. Substituting asparagine with serine in mouse at position 158 does not affect mouse PrP\textsuperscript{Sc} formation but substituting the same position in elk PrP affects elk PrP\textsuperscript{Sc} formation. This indicated these residues are species specific. Even when expressing the same amino acid in the same position, it shows a species specific role. Since this substitution does not affect mouse PrP\textsuperscript{Sc} formation, it would be useful to use this 5C6 epitope negative mouse PrP in the scrapie cell assay (Klohn, Stoltze et al. 2003; Bian, Napier et al. 2010). We could differentiate the wild type and mutated form by using our novel mAb 5C6.

MAb 9E9 has narrow specificity and only has strong affinity for cervid PrP. It has less affinity with horse and sheep PrP. It might be influenced by the amino acid serine after glycine at position 93, because in elk PrP and our immunogen shuffled PrP #68, there is threonine after glycine at position at 92. 9E9 epitope is adjacent to C2 cleavage site; an extra glycine in mouse PrP sequence interrupts the 9E9 recognition. It was confirmed when we deleted glycine in mouse PrP at position 93 or inserted one glycine at the same position in elk PrP. When we produced the 9E9 epitope by deleting one glycine in mouse PrP, it did not prevent formation of mouse PrP\textsuperscript{Sc} but decreased formation of di-glycosylated PrPs. Does the 9E9 tag in this mutated mouse PrP influence the PK digestion site? Several studies suggest that either helix 1 (residues 144–154) or the 90–145 part of the PrP protein plays a critical role in the conversion process to a β-sheet structure (Peretz, Williamson et al. 1997; Zhang, Stockel et al. 1997; Glockshuber,
It would be interesting to engineer a transgenic mouse with this 9E9-positive mouse PrP and inoculate with mouse prion. Inserting one glycine at position 93 in elk PrP affects elk PrP$^{Sc}$ formation. This indicated that the sequence between C1 and C2 cleavage sites might be involved in the conversion of PrP$^C$ to PrP$^{Sc}$.

The binding ability of mAb 7H11 is sterically affected by the secondary structure of PrP. It recognizes mouse, elk, deer, and sheep and has weak affinity to human PrP. It does not recognize bovine PrP. The binding capacity of 7H11 was affected by the presence of two N-linked glycans and the disulfide bond. According to these results, we supposed one of the epitopes of mAb 7H11 is between the two N-linked glycosylation sites, the other epitope is located 30 amino acids away in the upstream of the sequence. This indicated that 7H11 is also a conformation dependent mAb. We do not have more results to assure the exact recognition site of 7H11 at this stage.

MAb 9H9 has narrow species specificity since it only reacts with mouse and hamster PrP. We only know this antibody recognized the end of C-terminus PrP and it has mouse specificity. If we can clarify the exact residues, it could be a great tool to study mouse PrP.

To further understand the mechanism of conversion and characterize the structure of Prions, we generated an array of mutated PrPs with contiguous ten amino acids deletion constructs across the structured region of PrP (amino acids
107 to 230). This region contains the alpha-helix, beta-sheet, and post-translational modifications like glycosylation. These deletion constructs provide a way to map our novel monoclonal antibodies. The western blot analysis gave us the surprising result that the folded structure of wild type PrP is ultra-stable, even when we treated our sample with a detergent like SDS and ran our gel under denaturing conditions. If we deleted 10 amino acids within the c-terminal region, it caused instability in the structure and had effects on antibody recognition.

We created four novel mAbs and each of them has unusual and useful properties. We can study prion replication from these novel epitopes. They will be helpful in studying prion pathogenesis.
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