Characterization of Conformation-Dependent Prion Protein Epitopes*

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Background: Despite structural reorganization during disease, conformational prion protein epitopes remain undefined.

Results: We characterize the properties of conformational epitopes.

Conclusion: Conformational epitopes depend on a tertiary structure shared by normal and protease-resistant prion protein.

Significance: Our studies address how denatured conformational epitopes remain functional, provide structural insights into normal and disease-related prion protein, and expand options for epitope tagging.

SUMMARY

Whereas prion replication involves structural rearrangement of cellular prion protein (PrPc), the existence of conformational epitopes remains speculative and controversial, and PrP transformation is monitored by immunoblot detection of PrP27-30, a protease-resistant counterpart of pathogenic PrPSc. We now describe the involvement of specific amino acids in conformational determinants of novel monoclonal antibodies (mAbs) raised against randomly chimeric PrP. Epitope recognition of two mAbs depended on polymorphisms controlling disease susceptibility. Detection by one, referred to as PRC5, required alanine and asparagine at discontinuous mouse PrP residues 132 and 158, which acquire proximity when residues 126 to 218 form a structured globular domain. The discontinuous epitope of glycosylation-dependent mAb PRC7 also mapped within this domain at residues 154 and 185. In accordance with their conformational
Infectious transmission in prion diseases, which include epidemics of bovine spongiform encephalopathy (BSE), scrapie of sheep and goats, chronic wasting disease (CWD) of cervids, and human Creutzfeldt Jakob disease (CJD) involves conformational conversion of the prion protein (PrP), which exists in two physicochemically distinct isoforms. The cellular form, PrP\textsuperscript{C}, is monomeric, sensitive to protease treatment and soluble in detergents, while disease-associated PrP\textsuperscript{Sc}, is partially protease resistant, detergent insoluble, and prone to aggregation. During disease, exponential accumulation of PrP\textsuperscript{Sc} results from template-mediated conversion of PrP\textsuperscript{C} by PrP\textsuperscript{Sc}, leading to fatal, untreatable central nervous system neurodegeneration.

Mammalian PrP genes encode a ~250 amino acid residue translation product, processed by removal of 22-residue amino-terminal, and 23-residue carboxyl-terminal signal peptides. Cleavage of the latter facilitates addition of a glycosyl phosphatidylinositol moiety by which PrP is anchored to the cell surface (1). While the amino-terminus of PrP\textsuperscript{C} is largely unstructured, and contains a tandem array of five copper binding octapeptide repeats (2), the region encompassing amino acids 126–218 (mouse PrP numbering is used throughout, unless otherwise stated) consists of three \( \alpha \)-helices and two short sections forming a \( \beta \)-pleated sheet (3). In both PrP\textsuperscript{C} and PrP\textsuperscript{Sc}, asparagine (N)-linked oligosaccharides are attached at residues 180 and 196, and a disulphide bond is formed between cysteine residues 178 and 213. In most examples of prion disease, in vitro treatment of PrP\textsuperscript{Sc} with proteinase K (PK) results in cleavage of approximately 66 amino-terminal amino acids, and persistence of a protease-resistant core referred to as PrP\textsuperscript{27-30}. Cleavage of PrP\textsuperscript{Sc} following residue 88 results a similar 21 kDa carboxy-terminal fragment, referred to as C2, originally observed in the brains of patients with CJD (4), and subsequently shown to be calpain-dependent (5). PrP\textsuperscript{C} is cleaved between amino acids 110/111 to produce a 17 kDa C-terminal fragment referred to as C1 (4).

Because of the proteinaceous nature of prions, antibodies have been invaluable reagents for studying virtually all aspects of pathogenesis. The seminal observation that polyclonal antisera raised against PrP\textsuperscript{27-30} (6) also reacted with PrP in uninfected brains (7) was instrumental in establishing the precursor-product relationship between the cellular and scrapie isoforms. Subsequent attempts to isolate anti-PrP monoclonal antibodies (mAbs) were not without significant challenges (6), in large part because the host does not mount an inflammatory response during prion infection. The availability of Prnp\textsuperscript{0/0} mice in which the PrP gene was ablated (8), circumvented the underlying immune tolerance to PrP, allowing the generation of various anti-PrP mAbs (9).

Definition of structural epitopes requires X-ray crystallography of anti-PrP mAb – antigen complexes (10,11). Since this approach is laborious, ELISA-based peptide scanning has been the preferred method to map the approximate locations of epitopes. However, surprisingly few anti-PrP mAbs are amenable to this form of characterization, leading previous investigators to infer the existence of discontinuous PrP epitopes (12-17). To date, the involvement of specific amino acid residues in such hypothesized conformational epitopes has not been described. Moreover, this inference raises a conundrum (13), since all such antibodies bind PrP in Western blots, where PrP is defined by investigators as denatured (12,13,15,18-22). We therefore set out to gain a better understanding of the involvement of specific amino acid residue in discontinuous, conformation-dependent PrP epitopes. We used a directed molecular evolution approach (23) to create shuffled genes expressing novel PrP epitopes, and, by determining the reactivities of the resulting mAbs against a large panel of PrP primary structures and polymorphic variants thereof, we mapped, and confirmed by mutational
analysis, the involvement of specific residues in epitope binding and PrP<sup>C</sup> to PrP<sup>Sc</sup> conversion.

**EXPERIMENTAL PROCEDURES**

*Transgenic mice* – Transgenic (Tg) mice expressing mouse, deer, bovine, and sheep PrP were generated using the cosSHa.Tet cosmid vector (24). Tg mice expressing deer PrP with polymorphisms at residues 95 and 96, elk, and human PrP with valine (V) or methionine (M) polymorphisms at residue 129 (25), were generated using the MoPrP.Xho vector (26). Tg mice expressing horse PrP were produced using a modified MoPrP.Xho vector, referred to as pJB1, in which BsiWI and FseI sites replaced the XhoI site. All mice were maintained on an inbred Prnp<sup>0/0</sup> FVB background (FVB/Prnp<sup>0/0</sup>).

*Generation of chimeric PrP coding sequences by DNA shuffling* – One hundred ng of recombinant pCAGGS vectors harboring elk or mouse PrP coding sequences were amplified with Pfu polymerase (Stratagene, La Jolla, USA) using the following conditions: pre-denaturation 95°C/3 min; 95°C/40 sec; 60°C/40 sec; 72°C/60 sec. PCR products were mixed in an equal ratio, and 3 µg total DNA was incubated at 15°C for 10 min, followed by addition of 0.15 unit DNase I (Roche, Mannheim, Germany), and a further incubation at 15°C for 10 min. The reaction was terminated by adding 10 µl of stop solution and incubation at 96°C for 10 min. DNase I-digested DNA was separated on a 2 % low-temperature agarose gel (Invitrogen, Carlsbad, USA), and fragments in the range of 10 to 300 bp were excised and extracted in phenol/chloroform. For re-assembly of small DNA fragments by PCR, 30 µl of DNase I-digested DNA fragments, 5 µl of Pfu Ultra buffer, 1 µl of dNTP (Roche, Mannheim, Germany), and 1 µl of Pfu Ultra (Stratagene, La Jolla, USA) were mixed and the volume was adjusted to 50 µl with dH<sub>2</sub>O. The re-assembly PCR was performed for 45 cycles using the following conditions: 94°C/3 min; 94°C/30 sec; (27°C + 1°C/cycle)/1 min; 72°C/(1 min + 4 sec/cycle). The PCR product was maintained at 72°C for 10 min and then stored at 4°C. Reassembled DNA fragments were amplified using primers recognizing 5’ and 3’ ends of the open reading frame containing added EcoRV and BamHI sites for 35 cycles using the following conditions: pre-denaturation 94°C/3 min; 94°C/45 sec; 47°C with increases of 0.7°C/cycle 1 min; 72°C/1 min. PCR products were held at 72°C for 10 min. Shuffled DNA fragments were digested with BamHI and EcoRV (New England BioLabs, Ipswich, USA), purified and then inserted into pIRESpuro3 vector (Clontech, Mountain View, USA). Following transformation of bacteria to antibiotic resistance, colonies were screened for recombinant plasmids by PCR. Plasmids were extracted from clones containing the correct sized recombinants using a QIA miniprep kit (Qiagen, Valencia, USA), and plasmids were sequenced using a CEQ8000 (Beckman Coulter, Fullerton, USA).

*Preparation of purified recombinant PrP* - Selected shuffled DNA constructs were PCR amplified, and cloned into pET100/D-TOPO® (Invitrogen, Carlsbad, CA, USA). Transformed BL21 Star<sup>TM</sup> (DE3) (Invitrogen, Carlsbad, USA) were cultured until the OD value reached to 1.0 at 650 nm, and induced using isopropyl 1-thio-β-D-galactopyranoside (IPTG) (Sigma-Aldrich, St. Louis, USA) at a final concentration of 1 mM. Bacteria were harvested after 5 h incubation at 37°C. Bacterial pellets were resuspended and lysed with CelLytic solution (Sigma-Aldrich, St. Louis, USA) and centrifuged to obtain inclusion bodies, followed solubilization with CelLytic IB solution (Sigma-Aldrich, St. Louis, USA). Purification was performed using Ni-NTA column (Qiagen, Valencia, USA) according to the manufacturer’s instructions. Purified protein was refolded by dialysis in 20 mM NaAc buffer (pH, 4.5). The 6-histidine tag was removed by cleavage with enterokinase (Invitrogen, Carlsbad, USA). Protein content was determined by bicinechinonic acid (BCA) assay (Pierce Biotechnology, Rockford, USA), and purity was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining.

*Development and cloning of hybridoma cells* - FVB/Prnp<sup>0/0</sup> mice were immunized with 50 µg of purified recombinant PrP in Freund’s complete adjuvant by injection into the peritoneal cavity. Injections were repeated after 15, 30, and 45 days using 30 µg antigen with Freund’s incomplete adjuvant. Finally, 3 days prior to fusion, mice received the same amount of antigen in PBS without adjuvant. Mice with the highest antibody titer were used in cell fusions. Harvested spleen cells were fused with P3X63.AG8.
myeloma cells (ATCC, TIB-9) at a ratio of 2:1 in DMEM (HyClone, Logan, USA) with 50% (w/v) polyethylene glycol 1500 (Roche, Indianapolis, USA). Hybridoma cells were selected in medium containing hypoxanthine-aminopterin-thymidine (HAT).

Characterization of antibody reactivity by ELISA - ELISA plates (NUNC, Rochester, USA) were coated with 100 µl of 1 mg/ml recombinant shuffled PrP solution and stored overnight at 4°C. Plates were blocked with 200 ml 3% bovine serum albumin in PBS/0.05% Tween 20 for 2 hours at 37°C and washed three times with PBS/0.05% Tween 20. Plates were incubated with 100 µl conditioned medium from hybridoma cells for 2 h at 37°C. After three PBS-Tween 20 washes, 100 µl peroxidase-conjugated goat anti-mouse antibody (Sigma-Aldrich, St. Louis, USA) at a dilution of 1:5000 was added, and the plates were incubated for 1 h at 37°C, followed by three PBS-Tween 20 washes. Color development was accomplished by adding 100 ml of ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) peroxidase solution (KPL, Gaithersburg, USA) at room temperature in the dark. The color reaction was stopped by adding 100 µl of stop solution (KPL, Gaithersburg, USA), and the absorbance of samples was measured at 405 nm in an ELISA plate reader (ELx808, BioTek Instruments, Winooski, USA). Isotyping was performed by ELISA using isotype-specific anti-immunoglobulin conjugates (Pierce Biotechnology, Rockford, USA).

Antibody purification - Hybridoma cells were cultured in a 50/50 solution of complete DMEM and CDM4Mab (HyClone, Logan, USA), which is a serum-free medium for the culture of hybridoma cells. After one week the medium was collected and cell debris was removed by centrifugation at 2,000 x g for 10 min. Antibodies were purified by affinity chromatography using a HiTrap™ Protein G column (GE Healthcare, Piscataway, USA) and the Profinia™ protein purification system (Bio-Rad, Hercules, USA) with preprogrammed methods for antibody purification. Following equilibration of the Protein G column with 20 mM sodium phosphate, pH 7.0 binding buffer, hybridoma culture medium was applied at a rate of 1 ml/min. After washing the column with binding buffer, antibodies were eluted with 0.1 M glycine-HCl, pH 2.7 that was neutralized by the addition of 50 µl of 1 M Tris-HCl (pH 9.0) per 1 ml of elution buffer.

Stable transfection, and prion infection of cultured cells - PrP coding sequences with or without mAb epitope mutations were synthesized (GenScript, Piscataway, USA) with AflII and EcoRI restriction endonuclease recognition sites at the 5’ and 3’ ends respectively. Digested amplicons were inserted into AflII- and EcoRI-cleaved pRESpuro3 (Clontech, Mountain View, USA). PrP expression cassettes containing in-frame deletions were generated by PCR based mutagenesis using QuickChange mutagenesis kit (Stratagene, La Jolla, USA) according to the manufacturer’s instructions. Mutated constructs were sequenced using a CEQ 8000 (Beckman Coulter, Fullerton, USA). Rabbit kidney epithelial cells (RK13) were plated in 6-well plates one day prior to transfection. Transfection mixtures were prepared by mixing 2 µg of plasmid and 10 µl of lipofectamine 2000 (Invitrogen, Carlsbad, USA) in 500 µl of Opti-MEM medium (Invitrogen, Carlsbad, USA). After 5 h, the transfection solution was exchanged with complete medium containing 10% FBS, followed by passage to 10 cm plates the next day. Transfected cells were selected in complete medium containing 1 µg/ml puromycin.

For infection, transfected cells (2 x 10^5 cells/well) were plated in 6-well plates, and 0.2% brain homogenates in PBS were added to cell monolayers. After 5 h, 2 ml of complete medium was added, and cells were incubated for 5 days. After 3 passages, lysates of confluent cell monolayers were prepared in cold lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 0.5% Igepal CA-630) and analyzed by Western blotting.

Western blotting - Brain homogenates were prepared in 10% (w/v) sterile PBS lacking Ca<sup>2+</sup> and Mg<sup>2+</sup> by repeated extrusion through 18-gauge needles. Protein content in brain homogenates and cell lysates was determined by BCA (Pierce Biotechnology, Rockford, USA). Brain homogenates and cell lysates were digested with 100 µg/ml or 30 µg/ml of proteinase K (PK) respectively (Roche, Mannheim, Germany) in cold lysis buffer for 1 h at 37°C. Digestion was terminated with phenylmethylsulfonyl fluoride at a final
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Samples were prepared for SDS-PAGE either in the presence or absence of β-mercaptoethanol (βME) (Bio-Rad, Hercules, USA) and boiled for 10 min. Proteins were resolved by SDS-PAGE and transferred to polyvinylidenedifluoride Immobilon (PVDF)-FL membranes (Millipore, Billerica, USA). Membranes were probed with primary mAbs followed by horseradish peroxidase–conjugated anti-mouse secondary antibody (GE Healthcare, Little Chalfont, UK). Protein was visualized by chemiluminescence using ECL Plus (GE Healthcare, Piscataway, USA) and an FLA-5000 scanner (Fujifilm Life Science, Woodbridge, USA).

Surface Plasmon Resonance (SPR) measurements – SPR measurements were obtained with a Biacore T100 instrument (GE Healthcare, Uppsala, Sweden). The surfaces of flow channels on a CM5 chip were activated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS). Recombinant mouse PrP or elk PrP was diluted to 20 µg/ml in 10 mM sodium acetate (pH 5.5) and immobilized on a channel of the activated CM5 sensor chip. After immobilization, all surfaces were blocked with 1 M ethanolamine hydrochloride (pH 8.5). Another channel of the chip was processed through an identical coupling procedure without addition of ligand recombinant PrP and was used as reference. All kinetic SPR analyses were run at a 10 µl/min PBS flow, and antibodies were injected at concentrations ranging from 50 to 3200 nM. Association or dissociation was recorded for 180 s or 360 s, respectively. After each cycle, the surface was regenerated with 50 mM NaOH. Kinetic data were calculated using Biacore T100 evaluation software according to the bivalent analyte model.

Structural and statistical analyses – Three-dimensional structures of mouse PrP (3) were analyzed using the iMol Molecular Visualization Program, (2007) http://www.pirx.com/iMol. Statistical analyses were performed using Prism 5.0d for Mac OS X, (2010), http://www.graphpad.com, GraphPad Software Inc.

RESULTS

Generation of chimeric PrP by DNA shuffling - Nucleotides within the mouse Prnp and cervid PRNP gene coding sequences share ~80 % identity, and the primary structures of their translation products are ~85 % identical. We recombined the coding sequences of mouse and elk PrP in vitro, using a PCR-based technique referred to as DNA shuffling (27), to produce a library of randomly chimeric PrP. DNA sequence analysis of 96 recombinant clones identified 34 different shuffled PrP sequences. Three chimeras, referred to as Mo/CerPrP-3, Mo/CerPrP-34 and Mo/CerPrP-68 were selected for bacterial expression and recombinant (Rec) PrP purification.

Isolation of PRC monoclonal antibodies - High antibody titers resulted from immunization of Prnp0/0 mice with purified Rec PrP preparations. Following fusion of splenocytes from immunized mice with myeloma cells, we obtained two clones from Rec Mo/CerPrP-3 immunized mice, 25 clones from Rec Mo/CerPrP-68 immunized mice, and no clones from Rec Mo/CerPrP-34 immunized mice. Of the 25 clones from Rec Mo/CerPrP-68 immunized mice, 14 had reproducibly strong reactivity against Rec Mo/CerPrP-68 by ELISA and Western blotting. Ultimately, four mAbs, originally coded as 9E9, 5C6, 7H11, and 9H9 (28,29), hereafter referred to as PRC (Prion Research Center) 1, 5, 7, and 9 respectively, were selected for further characterization. Hybridomas were cloned two additional times. Monoclonal antibody PRC5 is IgG2a, while mAbs PRC1, PRC7, and PRC9 are IgG1.

Epitope characterization of PRC monoclonal antibodies - We determined the antigen binding properties of the PRC mAbs by probing Western blots of brain extracts from Tg mice expressing mouse, deer, cattle, sheep, horse, or human PrP, as well as various species including Syrian, Chinese and Armenian hamsters, mink, ferret, squirrel monkey, and cattle. Comparison of the reactive and non-reactive PrP primary structures with that of the shuffled Rec Mo/CerPrP-68 immunogen allowed us to identify specific amino acid residues that were critical for recognition by the various PRC mAbs. Of the 30 amino acid differences between mouse and elk PrP, recombinant Mo/CerPrP-68 shares 14 with...
PrP (Figs. 2, 6A, and 7).

addition, aglycosyl and monoglycosyl, but primary structures with a range of (Fig. 1C). No other amino acids differing between PRC7-reactive and non-reactive PrP primary structures correlated with antibody binding.

To confirm their role in the functional epitope of PRC5, we engineered constructs in which each of these two residues were individually mutated in mouse or elk PrP. Wild type and mutated constructs, referred to as MoPrP-V136, MoPrP-S158, ElkPrP-V133 and ElkPrP-S162, were stably expressed in RK13 cells, which do not express endogenous PrP (30). Consistent with a requirement for both residues in mAb recognition, mutation of either residue in each primary structure prevented detection by PRC5 (Fig. 1C).

While mAb PRC7 also detected a wide range of PrP primary structures, it failed to react with cattle, human, or hamster PrP (Fig. 2A). In addition, PRC7 preferentially reacted with aglycosyl and monoglycosyl, but not diglycosyl PrP (Figs. 2, 6A, and 7). While all reactive primary structures contain tyrosine (Y) at the position corresponding to residue 154 in mouse PrP, hamster species contain N, and cattle and human PrP contain histidine (H) (Fig. 2B). To confirm the requirement of this residue in recognition by PRC7, we changed Y at residue 154 in mouse PrP to N, and changed N at the corresponding residue in Syrian hamster PrP to Y. Mutant and wild type constructs were stably expressed in RK13 cells, and Western blotted extracts were probed with PRC7 (Fig. 2C). Consistent with a requirement for Y at residue 154 in mouse PrP in mAb recognition, mutation to N prevented detection by PRC7, while mutation of N to Y at position 155 in Syrian hamster PrP allowed detection by PRC7 (Fig. 2C). We also mutated H to Y at position 166 in cattle PrP, which corresponds to position 154 in mouse PrP, but this mutation failed to confer reactivity to PRC7 (Fig. 2C). Cattle PrP uniquely contains glutamic acid (E) at position 197 instead of glutamine (Q), which is conserved in all other species (Fig. 2B). To address whether this residue also partakes in PRC7 recognition, we changed the corresponding mouse PrP residue Q185 to E. This mutation, either singly, or in combination with N154, prevented recognition by PRC7, whereas reversal of both N to Y, and E to Q at the corresponding residues 166 and 197 in cattle PrP, allowed detection by PRC7. We conclude that mouse PrP residues 154 and 185 participate in PRC7 recognition. No other amino acid differences between PRC7-reactive and non-reactive PrP species correlated with antibody binding.

PRC1 reacted with deer, elk, cattle, horse, and sheep PrP, and failed to react with mouse (Fig. 3A and D) or human PrP (Fig. 3A). PRC1 also failed to react with deer PrP expressing histidine (H) at residue 95, referred to as deer PrP-H95 (deer PrP residue numbering). Immunodetection with mAb 6H4 (31) confirmed deer PrP-H95 transgene expression (Fig. 3A). The primary structures of deer PrP-H95 and wild type deer PrP expressing glutamine (Q) at 95 differ at only this single residue.

Conservation of Q at this position between reactive and non-reactive species (Fig. 3B) demonstrated that additional primary structural elements controlled PRC1 binding. While species that failed to react with PRC1 contained three consecutive G residues immediately following this Q, reactive species contained only two (Fig. 3B), suggesting that the number of G residues at this location controlled recognition by PRC1. To address this, we created mutated mouse and elk PrP coding sequences, referred to as MoPrP-2G and ElkPrP-3G, in which the number of G residues was changed from three to two in the mouse PrP sequence, and from two to three in elk PrP. Mutant and wild type constructs were stably expressed in RK13 cells and Western blotted extracts were probed with PRC1. Each construct had the expected reactivity with PRC1: deletion of one of

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the three G residues between 91 and 93 in mouse PrP conferred susceptibility to MoPrP-2G, while insertion of an additional G residue at the corresponding location in elk PrP abolished detection in ElkPrP-3G (Fig. 3C). Immunoblot probing with mAb 6H4 confirmed transgene expression in all cases. Consistent with this epitope location, PRC1 detected full-length elk PrP, C2, and PrP27-30, but failed to react with C1 following PNGaseF mediated removal of N-linked glycans; in contrast, mAb 6H4 detected full-length mouse and elk PrP, and both endogenously cleaved fragments (Fig. 3D).

Of the four PRC mAbs, PRC9 had the most restricted reactivity, responding only to mouse and hamster PrP (Fig. 4A). By comparing the PrP primary structures of Rec Mo/CerPrP-68 with those of reactive and non-reactive species, we located the PRC9 epitope within the C-terminus of PrP (Fig. 4B). Mouse and hamster PrP primary structures contain aspartic acid (D) and G residues at positions 226 and 227, while non-reactive species lacked these two adjacent residues. Expression of additional mouse PrP mutants in RK13 cells showed that PRC9 binding was inhibited by mutation of Y to A at residue 224, which is conserved in all tested species except horse, but not mutation of Y to A at residue 225 (Fig. 4B). Our results demonstrate that the PRC9 epitope overlaps the binding locations of recombinant R1 and R2 antibodies, which have been mapped to the region containing residues 224 to 230 (16).

Unexpected mAb reactivities with redacted PrP constructs - As an additional approach to map the locations of PRC mAb binding epitopes, we constructed a series of in-frame, non-overlapping deletions across the primary structure of mouse PrP between residues 107 and 230 (Fig. 5A). We expressed these redacted constructs in RK13 cells, and, following PNGaseF removal of N-linked sugars from PrP constructs, we probed cell extracts with various antibodies on Western blots. We expected that mAbs would fail to react with recombinant PrP deletions containing sequences comprising their epitopes. Based on our demonstration that PRC5 recognition involved mouse PrP residues 132 and 158, and PRC7 recognition involved mouse PrP residues 154 and 185, we therefore anticipated that PRC5 would fail to recognize PrP containing deletions between residues 132-141 and 152-161 and that PRC7 would fail to recognize PrP containing deletions between residues 152-161 and 182-191 (Fig. 5A). However, PRC5 and PRC7 unexpectedly either failed to detect, or had reduced reactivity with all constructs harboring deletions between residues 122 and 222, including those that were not predicted to harbor epitope components (Fig. 5B). This region corresponds to the globular, carboxyl-terminal domain which contains regions of secondary structure, defined by NMR analyses as being comprised of residues 216 to 218 (3) (Fig. 5A). PRC5 and PRC7 recognized all constructs harboring deletions outside this domain between 107-121, and 222-230 (Fig. 5B). In contrast to the behavior of PRC5 and 7, PRC9 satisfied our initial expectations, since it reacted with all deletion constructs except MoPrPΔ222-230, which contains its epitope (Fig. 5B). The reactivity of PRC9 also confirmed that compromised PRC5 and PRC7 binding to deletions between residues 122 and 222 was not the result of unstable mutant PrP expression in mammalian cells and bacteria. We obtained similar antibody reactivities when we expressed redacted constructs in bacteria, and probed cell extracts with antibodies on Western blots (Supplementary Fig. 1).

We next expanded our analyses to include previously characterized mAbs. The epitopes of 6H4 and D18 have previously been mapped to the region encompassing residues 132-156 of mouse PrP (Fig. 1B) (16,31). Similar to the behavior of PRC5 and PRC7, deletions affecting regions of secondary structure between residues 126 to 218, also prevented or reduced detection by mAbs 6H4, and D18, whereas deletions within unstructured regions had no effect on immunodetection (Fig. 5B). These findings supported the notion that failure of PRC5 and PRC7 to bind deletions within the structured domain was not the result of undiscovered epitope binding elements, but rather that deletion mutations within the PrP globular domain perturb the correct tertiary structure that region, and thus affect the topology of conformation-dependent epitopes of mAbs PRC5, PRC7, 6H4, and D18. In contrast to mAbs binding epitopes in the structured region, HumP, the epitope of which is adjacent to the PRC1 binding site (32) (Fig. 3B), and POM2, which binds mouse PrP at an adjacent location in the octapeptide repeat region (13), recognized all deletions and
wild type PrP (Fig. 5B and Supplementary Fig. 1), while R1 bound all constructs except MoPrPΔ222-230 which harbors its epitope (Fig. 5B).

Conformation-dependent antibody reactivities - The almost 30 residue separation between residues 132 and 158 demonstrates that the epitope recognized by PRC5 is discontinuous. The adjacent proximity of residues 132 and 158 in the tertiary structure of PrP (3) (Fig. 1B) therefore indicates that PRC5 reactivity is dependent on PrP conformation. In previous studies, the inability to map the locations of epitopes by peptide scanning, and reduced reactivity of the corresponding mAbs after disulfide bond reduction was cited as evidence that those epitopes were discontinuous, and therefore that recognition depended on intact PrP tertiary structure (15). In keeping with these previous observations, reactivity of mAb PRC5 with mouse and cervid PrP on Western blots was increased 1.5- (P < 0.05) and 1.7-fold (P < 0.005) in the absence of βME (Fig. 6A and B). Consistent with conformation-dependent reactivity of PRC7 involving discontinuous residues 154 and 185, immunodetection of mouse PrP by PRC7 was increased ~4-fold, and with cervid PrP ~12-fold (P < 0.01) under non-reducing compared to reducing Western blotting conditions (Fig. 6A and B). Surprisingly, since its binding has been mapped to a single sequential length of the polypeptide chain (31), mAb 6H4 reactivity with both cervid and mouse PrP was also increased ~3-fold (P < 0.01) under non-reducing conditions (Fig. 6A and B), indicating that its epitope also shares the property of conformational-dependence with the discontinuous epitopes of PRC5 and PRC7. In accordance with the notion that PRC1 recognizes a conformation-independent epitope, reactivity of cervid PrP with PRC1 was unaffected by treatment with reducing agents, whereas PRC9 immunoreactivity was enhanced 1.5-fold (P < 0.05) following treatment of mouse PrP with βME (Fig. 6A and B).

The conformational dependence of PRC5, PRC7, 6H4, and D18 recognition appeared incompatible with the ability of these mAbs to recognize PrP on Western blots, since PrP is generally regarded as denatured under these conditions (12,13,15,18,20-22). Furthermore, treatment of samples with guanidinium hydrochloride concentrations as high as 6M prior to SDS-PAGE and immunoblotting, failed to prevent PrP detection by PRC5 by Western blotting (Fig. 7C). To reconcile these discrepant observations, we hypothesized that, subsequent to denaturing treatments, PrP refolds during Western blotting to a structure amenable to detection by conformation-dependent antibodies. To address this, we denatured PrP bound to PVDF membrane following SDS-PAGE and Western blot transfer, by treatment with 100 mM βME/2% SDS at 55 °C for 30 min prior to immunoprobing with conformation-dependent or conformation-independent mAbs. In accordance with our expectations, recognition of mouse and elk PrP by the conformation-independent mAb POM2 (Supplementary Fig. 1) was unaffected by post-Western denaturation, while the reactivities of conformation-dependent antibodies PRC5, PRC7, and 6H4 with mouse or elk PrP were consistently reduced compared to membranes not receiving post-transfer denaturing treatments (Fig. 7).

Reactivities of PRC mAbs with mouse and cervid PrP under native conditions - Antibody binding of PrP expressed on the surface of RK13 cells was monitored by FACS (Supplementary Fig. 2A and B), and the ability of mAbs to react with PrP in solution was monitored by immunoprecipitation (Supplementary Fig. 2C). Measurements of the K_D values for mAb binding to immobilized mouse and elk PrP were determined by SPR (Table I). Consistent with its reactivity in Western blotting conditions, PRC5 efficiently recognized native mouse and elk PrP expressed on the surface of RK13 cells by FACS and immunoprecipitated mouse and elk PrP from brain homogenates of wild type and Tg(ElkPrP) mice respectively. Measurements of the K_D values for PRC5 binding to immobilized mouse and elk PrP by SPR, gave values of 2.1 x 10^-6 and 9.1 x 10^-7 respectively. PRC1 efficiently recognized elk but not mouse PrP by FACS and immunoprecipitation, and the K_D for binding to recombinant elk PrP was 7.0 x 10^-7. In contrast, PRC9 immunoprecipitated mouse but not elk PrP, and recognized mouse PrP expressed on the surface of RK13 cells, but less strongly than PRC5 or 6H4. Accordingly, the K_D for binding to mouse PrP was 1.2 x 10^-5. PRC7 also recognized cell surface expressed mouse and elk PrP, but less efficiently than PRC5. Reflecting this, K_D for PRC7 binding to elk PrP was 1.7 x 10^-4. PRC7 failed to immunoprecipitate either species of PrP.

Involvement of residues required for mAb
Conformation-dependent PrP epitopes

Having confirmed the roles of specific amino acid residues in the epitopes of different PRC mAbs, we investigated the effects of mutations at these positions on the ability of either elk or mouse PrP to be converted into PrPSc. To address this, we infected RK13 cells stably expressing wild type and mutated versions of mouse or elk PrP with the respective species of prions, and monitored the ability of mutated PrP to be converted to mouse or elk PrP27-30. The effects of mutations of various epitope components depended on whether they were expressed in the context of mouse or elk PrP. For PRC5, mutation of either 133A to V or 162N to S inhibited conversion of elk PrPSc to PrPSc (Fig. 8A). In contrast, while mutation of 132A to V also inhibited conversion of mouse PrPSc to PrPSc, mutation of 158N to S did not inhibit conversion of mouse PrPSc to PrPSc but instead augmented PrPSc levels (Fig. 8B). In the case of PRC7, while mutation of Q to E at mouse PrP residue 185 prevented conversion to PrP27-30, mutation of Y at residue 154 to N had no impact on the ability of PrPSc to form protease-resistant PrP (Fig. 8C). Variable numbers of G residues immediately distal to mouse PrP Q90 controlled PrP detection by PRC1 (Fig. 3). Insertion of an additional G residue at this location in elk PrP, which prevented detection by PRC1 (Fig. 3C), abolished conversion to PrPSc (Fig. 8D). In contrast, deletion of a G residue in mouse PrP, which facilitated detection by PRC1 (Fig. 3C), did not prevent conversion to PrPSc (Fig. 8E), and the resulting MoPrPSc-2G could be detected by PRC1 (Fig. 8F). The effects of various epitope mutations on the ability of mouse and elk PrP to be converted to PrP27-30 are summarized in Table I.

DISCUSSION

DNA shuffling to create chimeric PrP – We used a directed molecular evolution approach to create shuffled genes expressing novel PrP epitopes as immunogens. Our approach was based on previous studies which used DNA shuffling to produce chimeric envelope proteins capable of inducing neutralizing antibodies against all four dengue virus serotypes (33,34), and to optimize immunogenicity and protective efficacy of Venezuelan equine encephalitis, and type 1 human immunodeficiency virus envelope proteins (35,36). Our strategy resulted in the isolation of mAbs that recognized cervid, but not mouse PrP (PRC1); mouse, but not cervid PrP (PRC9); as well as antibodies that recognized both mouse and cervid PrP (PRC5 and PRC7). Not unexpectedly, all PRC mAbs had variable cross-reactivities with PrP from different species, although in the case of the PRC9, cross reactivity was restricted to hamster PrP. The properties of epitopes recognized by these newly developed mAbs, as well as the known properties of previously characterized mAbs used in these studies are detailed in Table I.

Characterization of discontinuous, conformation-dependent PrP epitopes – In previous studies, lack of mAb binding to peptide fragments in ELISA led investigators to infer the existence of discontinuous PrP epitopes (15,16). In the case of the IgM mAb 15B3, which has been used to selectively immunoprecipitate PrP aggregates in infectious (31), as well as a Tg mouse model of a genetically-programmed prion disease (37), three distinct peptide sequences were found to react with 15B3 (human PrP amino acids 142–148, 162–170, and 214–226) (31). However, the direct involvement of specific amino acid residues in this, or any other conformation-dependent PrP epitope, has not been previously reported. Moreover, 15B3, and possibly other purported PrPSc-specific mAbs (38), have been shown to interact with non-infectious as well as infectious PrP aggregates (39), and additional studies provide evidence that 15B3 interactions with such aggregates are nonspecific and paratope-independent (40).

Our epitope mapping studies show that the highly conserved A residue at mouse PrP position 132, as well as N at residue 158, contribute to recognition by mAb PRC5, while residues 154 and 185 are components of the PRC7 epitope. Since antibody paratopes are occupied at most by 15 to 22 amino-acid antigen residues (41), the ~30 residue separation between amino acid residues in each case unequivocally defines the epitopes of PRC5 and PRC7 as discontinuous.

Consistent with the conformational-dependence of these discontinuous epitopes, we show that PRC5 and PRC7 immunoreactivity is reliant on the integrity of the disulfide bond linking α-helices 2 and 3, which stabilizes the tertiary structure of PrP. Our mutational analyses showing that recognition by PRC5, PRC7, 6H4, and D18 is inhibited exclusively by deletions.
disrupting the structured globular region, are also consistent with our characterization of these epitopes as conformation-dependent. Our findings indicate that deletions invading any aspect of the structured globular domain, even sequence elements far removed from defined epitope components, destabilize the overall tertiary structure of the region, preventing correct epitope presentation for antibody recognition. In the case of mAb PRC5, A132 and N158 are adjacent in PrP tertiary structure at the base of the loop containing α helix 1, which is formed during antiparallel juxtaposition of β sheet regions 1 and 2 (Fig. 1B) (3). Disulfide bond disruption, or destabilizing deletion mutations, disrupt the overall tertiary structure of the globular region, which in turn preclude residues 132 and 158 from acquiring the required proximity for PRC5 epitope formation. A similar explanation accounts for the behavior of the discontinuous, conformation-dependent PRC7 epitope spanning residues 154 and 185.

Since its epitope has been mapped to the region containing sequential amino acids DYEDRYYRE corresponding to mouse PrP residues 143–151 (31), the behavior of mAb 6H4 indicates that linear, as well as discontinuous epitopes within the structured globular domain, exhibit conformational dependence, which is consistent with previously observed behaviors of this mAb (20,42). Previous studies showing loss of 6H4 immunoreactivity following PrP reduction and alkylation (22) were interpreted to mean that its epitope in α helix 1 became masked and therefore unavailable for detection. However, our results favor the alternate hypothesis that immunoreactivity of 6H4, and other mAbs with epitope components in this region, depends on correct local folding of α-helix 1, and moreover, that the sustained integrity of this secondary structural element is co-dependent on the overall tertiary structure of the globular domain.

In contrast to these conformation-dependent epitopes with the globular domain, the epitopes of PRC9, R1, HumP and POM2, located in relatively unstructured regions proximal and distal to the globular region, are independent of PrP conformation for their recognition, since their ability to recognize PrP is unaffected by structure-disrupting deletions, or by treatment with disulfide bond reducing agents.

Coincidentally, while deletion analysis has suggested a discontinuous epitope for anti-PrP mAb T2 (43), our results also indicate that mapping approaches relying exclusively on a lack of mAb binding to deletions within the structured domain of PrP (43), may lead to spurious interpretations of epitope locations.

Renaturation of conformational PrP epitopes - Our results shed light on previously reported difficulties surrounding anti-PrP mAb epitope mapping, which resulted in speculation that PrP may be idiosyncratically processed by antigen presenting cells (12), or to inferences that the epitopes of such mAbs are discontinuous, and/or that peptide fragments in this format are unable to adopt specific conformations required for antibody binding (12-17). This latter hypothesis was rejected as highly unlikely (13), since all such antibodies bind PrP in Western blots, where PrP is defined by investigators as denatured (12,13,15,18-22). Our discovery that mAbs PRC5 and PRP7, which recognize discontinuous, conformation-dependent epitopes, react with wild type PrP on Western blots was also surprising. However, our findings indicate that, subsequent to denaturing treatments, PrP readily refolds during, or after Western blotting to a structure amenable to detection by conformation-dependent antibodies. While it is widely assumed that discontinuous epitopes are generally irreversibly destroyed under the denaturing conditions used for SDS-PAGE, positive reactions on Western blots with antibodies recognizing discontinuous epitopes are not without precedent (44).

Since PRC5, PRC7 and 6H4 also react with immunoblotted PrP27-30, our results demonstrate that PK-resistant PrP must also maintain the structural determinants required for recognition by these conformation-dependent mAbs. Our findings therefore indicate that, following denaturation, PrPΔC and PrP27-30 renature into a common structure amenable to detection by conformation-dependent mAbs. However, recent studies using mass spectrometry analysis coupled with hydrogen-deuterium exchange indicate that PrPΔC consists of β-strands, relatively short turns and/or loops, and no α-helical structure, which represents a conformation radically different from PrPΔC(45). Our results therefore also suggest that the conformation of
renatured PrP27-30 is structurally more akin to PrP\textsuperscript{C} than to PrP\textsuperscript{Sc} from which it is derived. 

**PRC mAbs discriminate PrP** polymorphisms and PrP glycotypes - Epitopes of two of the four PRC mAbs are comprised of PrP residues that are polymorphic and influence disease susceptibility in sheep and deer. PRC5 reactivity is dependent on the sheep A/V polymorphism at residue 136. Susceptibility of sheep to classical scrapie is strongly associated with amino acid polymorphism at this residue, as well as polymorphisms at residues 154 and 171 (46). While previous studies have shown mAbs to be capable of distinguishing OvPrP-Q171 and OvPrP-R171 allotypes (47,48), the direct involvement of residue 171 in these epitopes was not demonstrated, and, in the case of glycosylation-dependent antibodies, the effect was indirectly influenced by N-linked glycan occupancy of OvPrP (47). Recognition by PRC1 is also dependent on a known PrP polymorphism. Detection of deer PrP was inhibited when H replaced Q at residue 95, but not when S replaced G at adjacent residue 96 (deer PrP numberings). These amino acid variations correspond to naturally occurring polymorphisms affecting susceptibility of white tail deer to CWD (49).

Monoclonal antibody PRC1 also fails to react with PrP primary structures containing three instead of two G residues immediately downstream of mouse PrP Q90. Our studies indicate that an additional G sterically hinders PRC1 recognition of its epitope, which includes Q at mouse PrP residue 90/deer PrP residue 95. Other than deer harboring the H95 polymorphism, monoclonal antibody PRC1 has excellent reactivity with cervid PrP, and this specificity is therefore likely to be useful for studies on CWD. Like other mAbs with epitopes located at the boundary between the octarepeat region and the protease-resistant core of PrP27-30 that have been used for molecular strain typing (13,50-52), PRC1 may be useful for differentiating biochemically distinct forms of PrP27-30 associated with different strains.

In addition to its conformational dependence, PRC7 immunoreactivity is also dependent on the state of glycosylation of PrP. Since PRC antibodies were derived from mice immunized with bacterially derived RecPrP, an N-linked glycan is unlikely to contribute to the PRC7 epitope. Instead, the preferential binding of PRC7 to under glycosylated PrP suggests that occupancy of one of the two PrP N-linked glycosylation sites precludes antibody binding to the fully glycosylated protein, making it immunologically silent to PRC7. A similar mechanism has been suggested to explain the properties of previously isolated glycosylation dependent antibodies (13,19,47).

**PRC mAbs expand the range of options for PrP epitope manipulation** – The involvement of specific amino acids in mAb recognition has, to date, only been defined for a handful of mAbs, in particular 3F4 (53) and L42 (54). One application for which such information has proved useful is epitope tagging of PrP molecules. Previous studies showed that PrP molecules engineered to include amino acid residues required for 3F4 binding could be converted to PrP\textsuperscript{Sc} in murine cell lines, chronically infected with adapted scrapie prions (24). While such an approach is useful for assessing the effects of amino acid alterations on PrP\textsuperscript{Sc} formation, it is not without drawbacks, since 3F4 epitope inclusion can lead to interfering effects on PrP\textsuperscript{Sc} formation (55). In addition, the exact composition of the 3F4 epitope has been recently debated (56). Stable expression of PRC epitope-mutated PrP constructs in RK13 cells not only allowed us to confirm the requirement of those residues for recognition by various PRC mAbs, but also to assess the effects of substitutions on conversion of PrP\textsuperscript{Sc}. Since RK13 cells support the replication of prions from a variety of species (29,30,57), we were able to assess the effects of these mutations on PrP\textsuperscript{Sc} formation in the context of multiple PrP primary structures. We found that mutation of amino acids constituting various PRC mAb epitopes had different effects on the abilities of mouse and cervid PrP to be converted to PrP\textsuperscript{Sc} (summarized in Table I), which is consistent with previous observations that structural requirements for PrP\textsuperscript{C} conversion to protease-resistant PrP vary between PrP primary structures (58). Such information expands the available options for introducing new, or eliminating existing PrP epitopes, while preserving the ability of mutated PrP\textsuperscript{C} to convert to PrP\textsuperscript{Sc}. For example, removal of one of three G residues after residue 90 preserved the ability of mutated mouse PrP to convert to PrP\textsuperscript{Sc}, and facilitated recognition by PRC1. Similarly,
mutation of mouse PrP at residues 158 and 154 preserved conversion to PrP\textsuperscript{Sc}, and abolished recognition by mAbs PRC5 and PRC7 respectively. Such manipulations allow, for example, newly converted PrP\textsuperscript{Sc} to be distinguished from PrP\textsuperscript{Sc} in the inoculum, and provide novel means of assessing the kinetics and cell biology of PrP\textsuperscript{Sc} formation. In conclusion, we used a directed molecular evolution approach to create shuffled genes expressing novel PrP epitopes as immunogens. To our knowledge, these studies are the first to describe the involvement of specific amino acids in functional, discontinuous, conformation-dependent anti-PrP mAb epitopes. We show that perturbations of the correct tertiary structure of the PrP globular domain, either by disulfide bond reduction, or by the introduction of conformation-disrupting deletion mutants, affect the topology of a variety of conformational epitopes. We also show that conformation-dependent PrP epitopes readily reform following conditions of harsh denaturation, and that both PrP\textsuperscript{C} and PrP\textsuperscript{27-30} re-nature into a structure amenable to detection by conformation-dependent mAbs. The epitopes of two of the four new antibodies described here are comprised of residues that are polymorphic in certain species, and affect disease susceptibility in those hosts. Because of their dependence on these polymorphisms for reactivity, PRC1 and PRC5 represent unique immunological tools to assess the conversion, and pathogenic effects of individual allotypes in animals co-expressing sheep PrP-A136/V136 and deer PrP-Q95/H95 (Saijo and Telling, manuscript in preparation). Finally, the mapping and mutational analyses of PrP mAb epitopes described here, not only expands the repertoire of available options for tagging PrP in Tg mice and transfected cell lines, but also provides new information about the role of these amino acids in conversion of PrP\textsuperscript{C} to PrP\textsuperscript{Sc}. 
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FOOTNOTES

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Abbreviations used are: PrP, prion protein; PrP<sup>C</sup>, cellular form of the prion protein; PrP<sup>Sc</sup>, scrapie form of the prion protein; mAb, monoclonal antibody; BSE, bovine spongiform encephalopathy; CWD, chronic wasting disease; CJD, Creutzfeldt Jakob disease; PK, proteinase K; Tg, transgenic; RK13, Rabbit kidney epithelial cells; *Prnp<sup>0/0</sup>*, mice in which the PrP coding sequence is disrupted
FIGURE LEGENDS

FIGURE 1. Functional epitope of mAb PRC5 A. Western blot reactivity with various PrP primary structures. AHa, Armenian hamster; Sq. Mon., squirrel monkey. B. Primary structures are aligned between mouse PrP residues 123 and 163. Reactive species are boxed and shaded in red. The 6H4 and D18 epitopes are bold in cattle and mouse PrP respectively. Residues 132 and 158 are boxed and shaded in blue, and side chains are yellow in the tertiary structure. C. Western blots of RK13 cell extracts transfected with the following: Vector, pIRESPuro; Mo wt, mouse PrP; Elk wt, elk PrP; Elk V133, elk PrP with V at residue 133; Elk S162, elk PrP with S at residue 162; Mo V132, mouse PrP with V at residue 132; and, Mo S158, mouse PrP with S at residue 158.

FIGURE 2. Functional epitope of mAb PRC7 A. Western blot reactivity with various PrP primary structures. Samples from hamsters, mink, ferret and squirrel monkey were prion infected. Syr., Syrian; Chin., Chinese; and Arm., Armenian hamsters; Sq. Mon., squirrel monkey. B. Primary structures are aligned between mouse PrP residues 152 and 198. Reactive species are boxed and shaded in red. Glycans attached at residues 180 and 196 are shown as green hexagons. Residues 154 and 185 are boxed and shaded in blue, and side chains are yellow in the tertiary structure. C. Western blots of RK13 cell extracts transfected with the following: Mo N154, mouse PrP with N at residue 154; Mo E185, mouse PrP with E at residue 185; Mo N154/E185, mouse PrP with N at residue 154, and E at residue 185; SHa wt, wild type Syrian hamster PrP; SHa Y155, Syrian hamster PrP with Y at residue 155; Bo wt, wild type cattle PrP; Bo Y166, cattle PrP with Y at residue 166; Bo Y166/Q197, cattle PrP with Y at residue 166, and Q at residue 197.

FIGURE 3. Functional epitope of mAb PRC1 A. Western blot reactivity with various PrP primary structures. Deer H95 and S96, deer PrP with H and S at residues 95 and 96 respectively. B. Primary structures are aligned between mouse PrP residues 81 and 115. Reactive species are boxed and shaded in red. The HumP and POM2 epitopes are bold in cattle and mouse PrP respectively. Amino acids controlling PRC1 reactivity are boxed and shaded in blue. Arrows depict the locations of protease cleavage to generate C2 and C1. C. Western blots of RK13 cell extracts transfected with the following: Vector, pIRESPuro; Mo wt, mouse PrP; Elk wt, elk PrP; Mo 2G, mouse PrP with two G residues after residue 90; Elk 3G, elk PrP with three G residues. D. Western blots showing the reactivity of mAb PRC1 with PrP in the brains of uninfected and RML prion infected wild type mice, and uninfected and CWD-infected Tg mice expressing elk PrP. Samples were treated with proteinase K (PK) or PNGaseF as indicated. Positions of unglycosylated full-length (F), C1, and C2 fragments are shown.

FIGURE 4. Functional epitope of mAb PRC9 A. Western blot reactivity with various PrP primary structures. B. Primary structures are aligned between mouse PrP residues 218 and 241. Reactive species are boxed and shaded in red. The R1 epitope is bold in hamster PrP. Amino acids controlling PRC9 reactivity are boxed and shaded in blue. Arrow depicts the GPI anchor signal peptidase cleavage site. C. Western blots of RK13 cell extracts transfected with the following: Vector, pIRESPuro; Mo wt, mouse PrP; Mo 224A and Mo 225A, mouse PrP harboring a mutation of Y to A at residue 224 and 225 respectively.

FIGURE 5. Effect of mAb recognition following PrP tertiary structure disruption by deletion mutagenesis A. Scheme showing the various deletion constructs and locations of mAb epitopes. Arrows or brackets indicate which deletions harbor epitope components for the various conformation-dependent mAbs. N-linked glycan attachment sites at residues 180 and 196 are indicated by green hexagons, and the disulphide bond between cysteine residues 178 and 213 as a red line. B. Western blot reactivity of conformation-dependent and -independent mAbs with PNGaseF-treated extracts of RK13 cells expressing PrP deletion constructs.
FIGURE 6. Effect of mAb recognition following PrP tertiary structure disruption by disulfide bond reduction A. Western blots showing relative immunoreactivities of mAbs with brain extracts of RML infected wild-type mice in the presence (+βME) or absence (-βME) of reducing agent. Samples treated with proteinase K (PK) as indicated. B. Densitometric analysis of mouse (blue) and elk (red) PrP levels in the presence (shaded bars) and absence (unshaded bars) of βME. *P<0.05, **P<0.005, ***P<0.001. O/R, ratio of oxidized to reduced PrP. C. Western blots demonstrating the effects of treatment with various concentrations of guanidinium hydrochloride on PrP detection in the brains of uninfected, or RML infected mice.

FIGURE 7. Immunoreactivity of conformation-dependent, but not conformation-independent mAbs is reduced following post-transfer denaturation of PrP. Brain extracts were separated by SDS-PAGE and transferred to PVDF-FL membranes. Membranes were then either not treated, or treated with 100 mM βME/2 % SDS at 55°C for 30 min prior to immunoprobing with the indicated antibodies.

FIGURE 8. Effects of mutating PRC mAb epitopes on PrPSc formation. Western blots of extracts from RK13 cells expressing wild type and mutant PrP, infected with CWD or RML prions A. and B. Residues involved in the PRC5 epitope. C. Residues involved in the PRC7 epitope. Mo N154, mouse PrP harboring mutation of Y to N at 154; Mo E185, mouse PrP harboring a mutation of Q to E at 185; Mo N154/E185, mouse PrP harboring mutations of Y to N at 154, and Q to E at 185. D., E. and F. Residues involved in the PRC1 epitope. Elk3G, elk PrP with three instead of two G residues; MoPrP2G, mouse PrP harboring two instead of three glycine residues; MoPrP2G/RML, infected with RML prions. Extracts were either treated (+) or not treated (-) with proteinase K (PK).
Supplementary Experimental Procedures
FACS
Immunoprecipitation
A. 

|        | Prnp<sup>0/o</sup> Mouse | Deer | Elk | Cattle | A136 | V136 | Horse | M129 | V129 |
|--------|--------------------------|------|-----|--------|------|------|-------|------|------|
| PRC5   | ![Image](https://example.com/PRC5.png) | ![Image](https://example.com/PRC5.png) | ![Image](https://example.com/PRC5.png) | ![Image](https://example.com/PRC5.png) | ![Image](https://example.com/PRC5.png) | ![Image](https://example.com/PRC5.png) | ![Image](https://example.com/PRC5.png) | ![Image](https://example.com/PRC5.png) | ![Image](https://example.com/PRC5.png) |

B. 

- **β1**
- **α1**
- **β2**

| Species        | Amino Acid Sequence |
|----------------|---------------------|
| Mouse          | GLGGYMLGSA<sup>130</sup>MSRPMIHFGND<sup>140</sup>WEDRYRE<sup>150</sup>NMYRY<sup>160</sup>PNQVYYR |
| Hamster        | GLGGYMLGSA<sup>130</sup>MSRPMHHFGND<sup>140</sup>WEDRYRE<sup>150</sup>NMRYPNQVYYR |
| Elk            | GLGGYMLGSA<sup>130</sup>MSRPLIHFGND<sup>140</sup>YEDRYRE<sup>150</sup>NMYRY<sup>160</sup>PNQVYYR |
| Deer           | GLGGYMLGSA<sup>130</sup>MSRPLIHFGND<sup>140</sup>YEDRYRE<sup>150</sup>NMYRY<sup>160</sup>PNQVYYR |
| Cattle         | GLGGYMLGSA<sup>130</sup>MSRPLIHFG<sup>140</sup>S<sup>150</sup>DYEDRYRE<sup>160</sup>NMYRY<sup>170</sup>PNQVYYR |
| Sheep (A136)   | GLGGYMLGSA<sup>130</sup>MSRPLIHFG<sup>140</sup>N<sup>150</sup>NDYEDRYRE<sup>160</sup>NHRYPNQVYYR |
| Sheep (V136)   | GLGGYMLGSA<sup>130</sup>MSRPLIHFG<sup>140</sup>N<sup>150</sup>DY<sup>160</sup>EDRYRE<sup>170</sup>NMRYPNQVYYR |
| Horse          | GLGGYMLGSA<sup>130</sup>MSRPIIHFG<sup>140</sup>NDYEDRYRE<sup>150</sup>NHRYPNQVYYR |
| Human          | GLGGYMLGSA<sup>130</sup>MSRPIIHFG<sup>140</sup>NS<sup>150</sup>DYEDRYRE<sup>160</sup>NHRYPNQVYYR |
| Mink           | GLGGYMLGSA<sup>130</sup>MSRPLIHFG<sup>140</sup>NDYEDRYRE<sup>150</sup>NHRYPNQVYYK |
| Ferret         | GLGGYMLGSA<sup>130</sup>MSRPLIHFG<sup>140</sup>NDYEDRYRE<sup>150</sup>NHRYPNQVYYK |
| Sq. Mon.       | GLGGYMLGSA<sup>130</sup>MSRPLIHFG<sup>140</sup>NDY<sup>150</sup>EDRYRE<sup>160</sup>NHRYPNQVYYR |
| Mo/CerPrP-68   | GLGGYMLGSA<sup>130</sup>MSRPMIHFGND<sup>140</sup>YEDRYRE<sup>150</sup>NHRYPNQVYYR |

C. 

|        | Vector | Mo wt | Elk wt | Elk V133 | Elk S162 | Elk V132 | Mo wt | Mo V133 | Mo S162 | Mo V132 | Mo S158 |
|--------|--------|-------|--------|----------|----------|----------|-------|----------|----------|----------|----------|
| PRC5   | ![Image](https://example.com/PRC5.png) | ![Image](https://example.com/PRC5.png) | ![Image](https://example.com/PRC5.png) | ![Image](https://example.com/PRC5.png) | ![Image](https://example.com/PRC5.png) | ![Image](https://example.com/PRC5.png) | ![Image](https://example.com/PRC5.png) | ![Image](https://example.com/PRC5.png) | ![Image](https://example.com/PRC5.png) | ![Image](https://example.com/PRC5.png) | ![Image](https://example.com/PRC5.png) |

Fig. 1
A. 

B. 

C. 

Fig. 2
A. 

| Prnp<sup>0/0</sup> | Mouse | Deer | Elk | Cattle | Sheep | Human |
|-------------------|-------|------|-----|--------|-------|-------|
|                   | A136  | V136 |     | M129   | V129  |       |
|                   | 36    | 29   |     | 19     | 36    |

B. 

|         | GQPHGGG-WGQGGTHSQWNKPSKPKTNLKHVAGAA |
|---------|-------------------------------------|
| Mouse   |                                    |
| Elk     |                                    |
| Deer    |                                    |
| Deer-S96|                                    |
| Deer-H95|                                    |
| Cattle  |                                    |
| Sheep   |                                    |
| Horse   |                                    |
| Human   |                                    |
| Mo/CerPrP-68|                            |

C. 

|         | PRC1 |
|---------|------|
| Deer    |      |
| Deer-H95|      |
| Deer-S96|      |

D. 

| Mouse | Mouse (RML) | Tg(ElkPrP) | Tg(ElkPrP) (CWD) |
|-------|-------------|------------|------------------|
|       |             |            |                  |
|       |             | F          |                  |
|       |             | C2         |                  |
|       |             | C1         |                  |

| PNGase | PK |
|--------|----|
| -      | -  |
| +      | +  |

**Fig. 3**
Fig. 4

A. 

|          | Mouse | Deer | Elk | Cattle | A136 | V136 | Horse | M129 | V129 |
|----------|-------|------|-----|--------|------|------|-------|------|------|
| Prnp<sup>0/0</sup> |       |      |     |        |      |      |       |      |      |
| PRC9     | 36    | 29   |     |        |      |      |       |      |      |

B. 

Mouse: VVEQMCVTQYQKESQAYMDDGRSSSTVLFSSPPV
Hamster: VVEQMCVTQYQKESQAYYDGRRSSSTVLFSSPPV
Elk: VVEQMCITQYQRESEAYYDGRRS-AVLFFSSPPV
Deer: VVEQMCITQYQRESEAYYDGRRS-AVLFFSSPPV
Cattle: VVEQMCITQYQRESEAYYDGRRS-AVLFFSSPPV
Sheep: VVEQMCITQYQRESEAYYDGRRS-AVLFFSSPPV
Horse: VVEQMCITQYQRESEAYYDGRRS-AVLFFSSPPV
Human: VVEQMCITQYQRESEAYYDGRRS-AVLFFSSPPV
Mink: VVEQMCVTQYQKESQAYYDGRRS-AVLFFSSPPV
Ferret: VVEQMCITQYQKESQAYYDGRRS-AVLFFSSPPV
Sq. Mon.: VVEQMCITQYQKESQAYYDGRRS-AVLFFSSPPV
Mo/CerPrP-68: VVEQMCITQYQKESQAYMDDGRSSSTVLFSSPPV

C. 

Vector

|          | Mo wt | Mo 224A | Mo 225A |
|----------|-------|---------|---------|
| PRC9     |       |         |         |
| PRC5     |       |         |         |
A.

![Diagram showing protein domains and segments](image)

B.

![Western blot images](image)

Fig. 5

Globular domain 126-218
**S. Fig. 2**

**A.**
- βME
- +βME

- **PRC7**
- **6H4**
- **PRC5**
- **PRC1**
- **PRC9**
- **PK**

**B.**

**Signal density (%)**

| mAb   | O/R ratio | PRC1 | 6H4 | PRC5 | PRC7 | PRC9 |
|-------|-----------|------|-----|------|------|------|
| PRC1  | 0.9       | ***  | *** | ***  | ***  | ***  |
| 6H4   | 2.8       |      | *** |      |      |      |
| PRC5  | 3.3       |      |     |      | ***  | ***  |
| PRC7  | 1.5       |      |     |      |      | ***  |
| PRC9  | 1.7       |      |     |      |      |      |

**C.**

**Mouse**
- GdnHCl [M]
- 0 2 4 6

**Mouse (RML)**
- GdnHCl [M]
- 0 2 4 6

**Signal**

- PRC5
- PRC7
- PRC9

Fig. 6
Fig. 7

No membrane denaturation

Membrane treatment with 100 mM βME/2% SDS @ 55°C for 30 min
Fig. 8
| mAb       | $K_D$ (M) | Constitutive amino acids | Linear/Discontinuous | Conformation dependent | Globular domain | Mutation Mouse PrP | Mutation Elk PrP | Effect of mutation on conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup> |
|-----------|-----------|---------------------------|----------------------|------------------------|-----------------|-------------------|------------------|---------------------------------------------------------------|
| **PRC mAbs** |           |                           |                      |                        |                 |                   |                   |                                                               |
| PRC1      | $7.0 \times 10^{-7}$ | -                         | Q90                  | Linear                 | No              | No                | PrP<sub>3G</sub> | Converts PrP<sub>3G</sub> | Inhibits PrP<sub>3G</sub> |                                    |
| PRC5      | $9.1 \times 10^{-7}$ | $2.1 \times 10^{-6}$       | A132 N158            | Discontinuous          | Yes             | Yes               | A132V N158S     | Converts PrP<sub>2G</sub> | Inhibits PrP<sub>2G</sub> |                                    |
| PRC7      | $1.7 \times 10^{-4}$ | ND                        | Y154 Q185            | Discontinuous          | Yes             | Yes               | Y154N Q185E     | Converts PrP<sub>7</sub> | Inhibits PrP<sub>7</sub> |                                    |
| PRC9      | -         | $1.2 \times 10^{-6}$       | Y224, D226, G227     | Linear                 | No              | No                | ND               | ND                                             |                                                   |
| **Previously reported mAbs** |         |                           |                      |                        |                 |                   |                   |                                                               |
| 6H4       | Unknown - within 143-151 | Linear                  | Yes                  | Yes                   |                 |                   |                   |                                                               |
| D18       | Unknown - within 132-156 | Discontinuous            | Yes                  | Yes                   |                 |                   |                   |                                                               |
| R1        | Unknown - within 224-230 | Linear                  | No                   | No                    |                 |                   |                   |                                                               |
| HumP      | Unknown - within 95-107 | Linear                  | No                   | No                    |                 |                   |                   |                                                               |
| POM2      | Unknown - within 81-89  | Linear                  | No                   | No                    |                 |                   |                   |                                                               |
Characterization of Conformation-Dependent Prion Protein Epitopes
Hae-Eun Kang, Chu Chun Weng, Eri Saijo, Vicki Saylor, Jifeng Bian, Sehun Kim, Laylaa Ramos, Rachel Angers, Katie Langenfeld, Vadim Khaychuk, Carla Calvi, Jason Bartz, Nora Hunter and Glenn C. Telling

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